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(54) Title: **ULTRA-SENSITIVE DETECTION SYSTEMS**

(57) Abstract: Disclosed are compositions and methods for sensitive detection of one or multiple analytes. In general, the methods involve the use of special label components, referred to as reporter signals, that can be associated with, incorporated into, or otherwise linked to the analytes. In some embodiments, the reporter signals can be altered such that the altered forms of different reporter signals can be distinguished from each other. In some embodiments, sets of reporter signals can be distinguished from each other. In some embodiments, sets of reporter signals can be used where two or more of the reporter signals in a set have one or more common properties that allow the reporter signals having the common property to be distinguished and/or separated from other molecules lacking the common property. In other embodiments, sets of reporter signal/analyte conjugates can be used where two or more of the reporter signal/analyte conjugates in a set have one or more common properties that allow the reporter signal/analyte conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. Reporter signals can also be in conjunction with analytes (such as in mixtures of reporter signals and analytes), where no significant physical association between the reporter signals and analytes occurs; or alone, where no analyte is present.

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ULTRA-SENSITIVE DETECTION SYSTEMS

FIELD OF THE INVENTION

This invention is generally in the field of detection of analytes and biomolecules, and more specifically in the field of multiplex detection and analysis of analytes and biomolecules.

BACKGROUND OF THE INVENTION

Detection of molecules is an important operation in the biological and medical sciences. Such detection often requires the use of specialized label molecules, amplification of a signal, or both, because many molecules of interest are present in low quantities and do not, by themselves, produce detectable signals. Many labels, labeling systems, and signal amplification techniques have been developed. For example, nucleic acid molecules and sequences have been amplified and/or detected using polymerase chain reaction (PCR), ligase chain reaction (LCR), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA), and amplification with Q β replicase (Birkenmeyer and Mushahwar, *J. Virological Methods*, 35:117-126 (1991); Landegren, *Trends Genetics* 9:199-202 (1993)). Proteins have been detected using antibody-based detection systems such as sandwich assays (Mailini and Maysef, "A sandwich method for enzyme immunoassay. I. Application to rat and human alpha-fetoprotein" *J. Immunol. Methods* 8:223-234 (1975)) and enzyme-linked immunosorbent assays (Engvall and Perlmann, "Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin " *Immunochemistry* 8:871-874 (1971)), and two-dimensional (2-D) gel electrophoresis (Patton, *Biotechniques* 28: 944-957 (2000)). Although these techniques are useful, most have significant drawbacks and limitations. For example, radioactive labels are dangerous and difficult to handle, fluorescent labels have limited capacity for multiplex detection because of limitations on distinguishable labels, and amplification methods can be subject to spurious signal amplification. There is a need for improved detection labels and detection techniques that can detect minute quantities of specific molecules and that can be highly multiplexed.

Analysis of protein expression and presence, such as proteome profiling or proteomics, requires sensitive detection of multiple proteins. Current methods in proteome profiling suggests that there is a shortage of tools necessary for such

detection (Haynes and Yates, *Proteome profiling-pitfalls and progress*. Yeast 17(2):81-87 (2000)). While the techniques of chromatography and capillary electrophoresis are amenable to proteomic studies and have seen significant development efforts (see for example, Krull *et al.*, *Specific applications of capillary electrochromatography to biopolymers, including proteins, nucleic acids, peptide mapping, antibodies, and so forth*. J Chromatogr A, 887:137-63 (2000), Hage, *Affinity chromatography: a review of clinical applications*. Clin Chem, 45(5):593-615 (1999), Hage *et al.*, *Chromatographic Immunoassays*, Anal Chem, 73(07):198 A-205 A, (2001), Krull *et al.*, *Labeling reactions applicable to chromatography and electrophoresis of minute amounts of proteins*. J Chromatogr B Biomed Sci Appl, 699:173-208 (1997)), the workhorse of the industry remains two dimensional electrophoresis where the two dimensions are isoelectric focusing and molecular size. Haynes and Yates point out the significant shortcomings of the technique but discuss the utility of the method in light of such shortcomings. Hayes and Yates also discuss the techniques of Isotope Coded Affinity Tags (ICAT), LC-LC-MS/MS, and stable isotope labeling techniques (Shevchenko *et al.*, *Rapid 'de novo' peptide sequencing by a combination of nanoelectrospray, isotopic labeling and a quadrupole/time-of-flight mass spectrometer*. Rapid Commun Mass Spectrom 11(9):1015-1024 (1997); Oda *et al.*, *Accurate quantitation of protein expression and site-specific phosphorylation*. Proc Natl Acad Sci U S A 96(12):6591-6596 (1999)).

Aebersold *et al.* (WO 00/11208) have described labels of the composition PRG-L-A, where PRG is a protein reactive group, L is a linker (that may contain isotopically distinguishable composition), and A is an affinity moiety. Aebersold *et al.* describes a method where the protein reactive group is used to attach the label to a protein, an affinity capture molecule is used to capture the affinity moiety, the remaining proteins are discarded, then the affinity moiety is released and the labeled proteins are detected by mass spectrometry. The method of Aebersold *et al.* does not involve fragmentation or other modification of the labels or proteins.

The technique of ICAT, where cysteine residues are labeled with heavy or light tags that each contain affinity moieties, in control and tester samples, has received significant interest and holds potential for protein profiling (Gygi *et al.*, *Quantitative analysis of complex protein mixtures using isotope-coded affinity tags*. Nat. Biotechnol.

17(10):994-999 (1999), Griffin *et al.*, *Quantitative proteomic analysis using a MALDI quadrupole time-of-flight mass spectrometer.*, Anal. Chem., 73:978-986 (2001)). Gygi *et al.* and Griffin *et al.* have demonstrated relative profiling of two protein samples, where the two samples are distinguished utilizing linkers containing either eight normal
5 hydrogen or eight heavy hydrogen (deuterium) atoms. The relative concentrations of labeled proteins are determined by ratio of peaks that are separated by the corresponding 8 amu difference in the linker molecules. Current implementations have been limited to two labels. This technique does not involve fragmentation or other modification of the labels or proteins.

10 Mass spectrometry has been used to detect phosphorylated proteins (DeGnove and Qin, *Fragmentation of phosphopeptides in an ion trap mass spectrometer.* J. Am. Soc. Mass Spectrom. 9:1175-1188 (1998); Qin and Chait, *Identification and characterization of posttranslational modifications of proteins by MALDI ion trap mass spectrometry.* Anal Chem, 69:4002-9 (1997); Annan *et al.*, *A multidimensional*
15 *electrospray MS-based approach to phosphopeptide mapping.* Anal. Chem. 73:393-404 (2001)). The methods make use of a signature mass to indicate the presence of a phosphate group, for example $m/z = 63$ and/or $m/z = 79$ corresponding to PO_2^- and PO_3^- ions in negative ion mode, or the neutral loss of 98 Daltons from the parent ion indicates the loss of H_3PO_4 from the phosphorylated peptide, indicate phosphorylated
20 Ser, Tyr, Thr. Once phosphorylated amino acids are identified, the peptide containing the modification is sequenced by standard MS/MS techniques. There is a need for a high reliability, highly multiplexed readout system for proteomics.

The status of any living organism may be defined, at any given time in its lifetime, by the complex constellation of proteins that constitute its "proteome." While
25 the complete status of the proteome could be defined by listing all proteins present (including modified variants) as well as their intracellular locations and concentrations, such a task is beyond the capabilities of any current single analytical method. However, attempts have been made to define the status of a cell or tissue by identifying and measuring the relative concentrations of a small subset of proteins. For example,
30 Conrads *et al.*, *Analytical Chemistry*, 72:3349-3354 (2000), have described the use of "Accurate Mass Tags" (AMT) for proteome-wide protein identification. Conrads *et al.* show, for a simple organism, that a mass spectrometer of sufficient mass accuracy and

resolution can be used to detect certain tryptic digest fragments from proteins. Once identified, the AMTs may be directly detected in samples by tryptic digest of the proteins, and high accuracy, high resolution mass spectrometry.

While the concept of Accurate Mass Tags is useful for protein discovery, as well as for generating peptide patterns in conventional biological experiments, it does not solve the problem of sensitivity that is at the heart of a truly useful diagnostic multi-protein assessment. A useful assessment consisting of AMTs will require samples containing a minimum of 2000 to 10,000 cells in order to permit reliable readout. This is so because many important cellular proteins are present at levels of only 500 to 5000 molecules per cell. If a clinically relevant protein is present in 500 copies per cell, and a precious clinical sample from a cancer patient contains only 1000 cells, the total number of proteins is 500,000, an amount that lies below the limit of detection by conventional mass spectrometry. Thus, the types of measurements proposed by Conrads et al. for the study of proteomes after identification of AMTs are not suitable for addressing important clinical problems such as the diagnosis of cancer.

BRIEF SUMMARY OF THE INVENTION

Disclosed are compositions and methods for sensitive detection of one or multiple analytes. In general, the methods involve the use of special label components, referred to as reporter signals, that can be associated with, incorporated into, or otherwise linked to the analytes. Reporter signals can also be used merely in conjunction with analytes, with no significant association between the analytes and reporter signals. Compositions where reporter signals are associated with, incorporated into, or otherwise linked to the analytes are referred to as reporter signal/analyte conjugates. Such conjugates include reporter signals associated with analytes, such as a reporter signal probe hybridized to a nucleic acid sequence; reporter signals covalently coupled to analytes, such as reporter signals linked to proteins via a linking group; and reporter signals incorporated into analytes, such as fusions between a protein of interest and a peptide reporter signal.

In some embodiments, the reporter signals can be altered such that the altered forms of different reporter signals can be distinguished from each other. Reporter signal/analyte conjugates can be altered, generally through alteration of the reporter signal portion of the conjugate, such that the altered forms of different reporter signals,

altered forms of different reporter signal/analyte conjugates, or both, can be distinguished from each other. Where the reporter signal or reporter signal/analyte conjugate is altered by fragmentation, any, some, or all of the fragments can be distinguished from each other, depending on the embodiment. For example, where
5 reporter signals fragmented into two parts, either or both parts of the reporter signals can be distinguished. Where reporter signal/analyte conjugates are fragmented into two parts (with the break point in the reporter signal portion), either the reporter signal fragment, the reporter signal/analyte fragment, or both can be distinguished. In some embodiments, only one part of a fragmented reporter signal will be detected and so
10 only this part of the reported signals need be distinguished.

In some embodiments, sets of reporter signals can be used where two or more of the reporter signals in a set have one or more common properties that allow the reporter signals having the common property to be distinguished and/or separated from other molecules lacking the common property. In other embodiments, sets of reporter
15 signal/analyte conjugates can be used where two or more of the reporter signal/analyte conjugates in a set have one or more common properties that allow the reporter signal/analyte conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. In still other embodiments, analytes can be fragmented (prior to or following conjugation) to
20 produce reporter signal/analyte fragment conjugates (which can be referred to as fragment conjugates). In such cases, sets of fragment conjugates can be used where two or more of the fragment conjugates in a set have one or more common properties that allow the fragment conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. It should be
25 understood that fragmented analytes can be considered analytes in their own right. In this light, reference to fragmented analytes is made for convenience and clarity in describing certain embodiments and to allow reference to both the base analyte and the fragmented analyte.

As indicated above, reporter signals conjugated with analytes can be altered
30 while in the conjugate and distinguished. Conjugated reporter signals can also be dissociated or separated, in whole or in part, from the conjugated analytes prior to their alteration. Where the reporter signals are dissociated (in whole or in part) from the

analytes, the method can be performed such that the fact of association between the analyte and reporter signal is part of the information obtained when the reporter signal is detected. In other words, the fact that the reporter signal may be dissociated from the analyte for detection does not obscure the information that the detected reporter signal was associated with the analyte.

Reporter signals can also be in conjunction with analytes (such as in mixtures of reporter signals and analytes), where no significant physical association between the reporter signals and analytes occurs; or alone, where no analyte is present. In such cases, where reporter signals are not or are no longer associated with analytes, sets of reporter signals can be used where two or more of the reporter signals in a set have one or more common properties that allow the reporter signals having the common property to be distinguished and/or separated from other molecules lacking the common property.

Detection of the reporter signals indicates the presence of the corresponding analytes. The reporter signals preferably can have two key features. First, the reporter signals can be used in sets where all the reporter signals in the set have similar properties (for example, mass spectrometry reporter signals may have similar mass-to-charge ratios). The similar properties allow the reporter signals to be distinguished and/or separated from other molecules lacking one or more of the properties. Second, all the reporter signals in a set can be fragmented, decomposed, reacted, derivatized, or otherwise modified to distinguish the different reporter signals in the set. Preferably, mass spectrometry reporter signals are fragmented to yield fragments of similar charge but different mass.

Differential distribution of mass in the fragments of the reporter signals can be accomplished in a number of ways. For example, reporter signals of the same nominal structure (for example, peptides having the same amino acid sequence) can be made with different distributions of heavy isotopes, such as deuterium; reporter signals of the same nominal structure can be made with different distributions of modifications, such as methylation, phosphorylation, sulphation, and use of seleno-methionine for methionine; reporter signals of the same nominal composition (for example, made up of the same amino acids) can be made with different ordering of the subunits or components of the reporter signal; and reporter signals having the same nominal

composition can be made with a labile or scissile bond at a different location in the reporter signal. Each of these modes can be combined with each other and/or one or more of the other modes to produce differential distribution of mass in the fragments of the reporter signals.

5 The reporter signals are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge ratios. The disclosed reporter signals can be used as general labels in myriad labeling and/or detection techniques. A set of isobaric reporter signals can be used for multiplex labeling and/or detection of many analytes since the reporter signal fragments can be
10 designed to have a large range of masses, with each mass individually distinguishable upon detection.

 The disclosed method has advantageous properties which can be used as a detection system in a number of fields, including antibody or protein microarrays, DNA microarrays, expression profiling, comparative genomics, immunology, diagnostic
15 assays, and quality control.

A. Reporter Molecule Labeling

 In one form of the disclosed method, referred to as reporter molecule labeling (RML), reporter signals are first associated with the analytes and then dissociated and detected. The reporter signals preferably are associated with the analytes via
20 interaction of specific binding molecules with the analytes. The reporter signals are either directly or indirectly associated with the specific binding molecules such that interaction of the specific binding molecules with the analytes allows the reporter signals to be associated with the analytes. The method can be performed such that the fact of association between the analyte and reporter signal is part of the information
25 obtained when the reporter signal is detected. In other words, the fact that the reporter signal may be dissociated from the analyte for detection does not obscure the information that the detected reporter signal was associated with the analyte.

B. Reporter Signal Labeling

 In another form of the disclosed method, referred to as reporter signal labeling (RSL), reporter signals are used for sensitive detection of one or multiple analytes. In
30 the method, analytes labeled with reporter signals are analyzed using the reporter signals to distinguish the labeled analytes (where the analytes are labeled with the

reporter signals). Detection of the reporter signals indicates the presence of the corresponding analyte(s). The detected analyte(s) can then be analyzed using known techniques. The labels provide a unique analyte/label composition that can specifically identify the analyte(s). This is accomplished through the use of the specialized reporter
5 signals as the labels. The labeled analyte(s) can be fragmented prior to analysis. An analyte sample to be analyzed can also be subjected to fractionation or separation to reduce the complexity of the samples. Fragmentation and fractionation can also be used together in the same assay. Such fragmentation and fractionation can simplify and extend the analysis of the analytes.

10 Reporter signals can be coupled or directly associated with an analyte. For example, a reporter signal can be coupled to an analyte via reactive groups, or a reporter molecule (composed of a specific binding molecule and a reporter signal) can be associated with an analyte. The reporter signals can be attached to analytes in any manner. For example, reporter signals can be covalently coupled to proteins through a
15 sulfur-sulfur bond between a cysteine on the protein and a cysteine on the reporter signal. Many other chemistries and techniques for coupling compounds to analytes are known and can be used to couple reporter signals to analytes. For example, coupling can be made using thiols, epoxides, nitriles for thiols, NHS esters, isothiocyanates for amines, and alcohols for carboxylic acids. Reporter signals can be attached to analytes
20 either directly or indirectly, for example, via a linker.

Alternatively, a reporter signal can be associated with an analyte indirectly. In this mode, a "coding" molecule containing a specific binding molecule and a coding tag can be associated with the analyte (via the specific binding molecule). Alternatively, a coding tag can be coupled or directly associated with the analyte. Then a reporter
25 signal associated with a decoding tag (such a combination is another form of reporter molecule) is associated with the coding molecule through an interaction between the coding tag and the decoding tag. An example of this interaction is hybridization where the coding and decoding tags are complementary nucleic acid sequences. The result is an indirect association of the reporter signal with the analyte. This mode has the
30 advantage that all of the interactions of the reporter signals with the coding molecule can be made chemically and physically similar by using the same types of coding tags and decoding tags for all of the coding molecules and reporter molecules in a set.

Reporter signals can be fragmented, decomposed, reacted, derivatized, or otherwise modified, preferably in a characteristic way. This allows an analyte to which the reporter signal is attached to be identified by the correlated detection of the labeled analyte and one or more of the products of the labeled analyte following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal (the
5 labeled analyte is the analyte/reporter signal combination). The alteration of the reporter signal will alter the labeled analyte in a characteristic and detectable way. Together, the detection of a characteristic labeled analyte and a characteristic product of the labeled analyte can uniquely identify the analyte. In this way, using the
10 disclosed method and materials, one or more analytes can be detected, either alone or together (for example, in a multiplex assay). Further, one or more analytes in one or more samples can be detected in a multiplex manner. Preferably, for mass spectrometry reporter signals, the reporter signals are fragmented to yield fragments of similar charge but different mass.

15 In preferred embodiments, reporter signals are used in sets where all the reporter signals in the set have similar properties (such as similar mass-to-charge ratios). The similar properties allow the reporter signals to be distinguished and/or separated from other molecules lacking one or more of the properties. Alternatively, or in addition, reporter signals can be used in sets such that the resulting labeled analytes
20 will have similar properties allowing the labeled analytes to be distinguished and/or separated from other molecules lacking one or more of the properties.

Analytes can be detected using the disclosed reporter signals in a variety of ways. For example, the analyte and attached reporter signal can be detected together, one or more fragments of the analyte and the attached reporter signal(s) can be detected
25 together, the fragments of the reporter signal can be detected, or a combination. Preferred detection involves detection of the analyte/reporter signal both before and after fragmentation of the reporter signal.

A preferred form of the disclosed method involves correlated detection of the reporter signals both before and after fragmentation of the reporter signal. This allows
30 labeled analytes to be detected and identified via the change in labeled analyte. That is, the nature of the reporter signal detected (non-fragmented versus fragmented) identifies the analyte as labeled. Where the analytes and reporter signals are detected by mass-to-

charge ratio, the change in mass-to-charge ratio between fragmented and non-fragmented samples provides the basis for comparison. Such mass-to-charge ratio detection is preferably accomplished with mass spectrometry.

As an example, an analyte in a sample can be labeled with reporter signal
5 designed as a mass spectrometry label. The labeled analyte can be subjected to mass spectrometry. A peak corresponding to the analyte/reporter signal will be detected. Fragmentation of the reporter signal in a collision cell in the mass spectrometer would result in a shift in the peak corresponding to the loss of a portion of the attached
10 reporter signal, the appearance of a peak corresponding to the lost fragment, or a combination of both events. Significantly, the shift observed will depend on which reporter signal is on the analyte since different reporter signals will, by design, produce fragments with different mass-to-charge ratios. The combination event of detection of the parent mass-to-charge (with no collision gas) and the mass-to-charge corresponding to the loss of the fragment from the reporter signal (with collision gas) indicates a
15 labeled analyte. The identity of the analyte can be determined by standard mass spectrometry techniques, such as compositional analysis.

A powerful form of the disclosed method is use of analytes labeled with reporter signals to assay multiple samples (for example, time series assays or other comparative analyses). Knowledge of the temporal response of a biological system
20 following perturbation is a very powerful process in the pursuit of understanding the system. To follow the temporal response, a sample of the system is obtained (for example, cells from a cell culture, mice initially synchronized and sacrificed) at determined times following the perturbation. Knowledge of spatial analyte profiles (for example, relative position within a tissue section) is a very powerful process in the
25 pursuit of understanding the biological system.

In the disclosed method a series of samples can each be labeled with a different reporter signal from a set of reporter signals. Preferred reporter signals for this purpose would be those using differentially distributed mass. In particular, the use of stable isotopes is preferred to ensure that members of the set of reporter signals would behave
30 chemically identically and yet would be distinguishable.

The labeled analytes are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge ratios.

The disclosed reporter signals can be used as general labels in myriad labeling and/or detection techniques. A set of isobaric reporter signals can be used for multiplex labeling and/or detection of many analytes since the reporter signal fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection. Where the same analyte or type of analyte is labeled with a set of isobaric reporter signals (by, for example, labeling the same analyte in different samples), the set of labeled analytes that results from use of an isobaric set of reporter signals will also be isobaric. Fragmentation of the reporter signals will split the set of labeled analytes into individually detectable labeled proteins of characteristically different mass.

The disclosed method can be used in many modes. For example, the disclosed method can be used to detect a specific analyte (in a specific sample or in multiple samples) or multiple analytes (in a single sample or multiple samples). In each case, the analyte(s) to be detected can be separated either from other, unlabeled analytes or from other molecules lacking a property of the labeled analyte(s) to be detected. For example, analytes in a sample can be generally labeled with reporter signals and some analytes can be separated on the basis of some property of the analytes. For example, the separated analytes could have a certain mass-to-charge ratio (separation based on mass-to-charge ratio will select both labeled and unlabeled analytes having the selected mass-to-charge ratio). As another example, all of the labeled analytes can be distinguished and/or separated from unlabeled molecules based on a feature of the reporter signal such as an affinity tag. Where different affinity tags are used, some labeled analytes can be distinguished and/or separated from others. Reporter signal labeling allows profiling of analytes and cataloging of analytes.

In one mode of the disclosed method, multiple analytes in multiple samples are labeled where all of the analytes in a given sample are labeled with the same reporter signal. That is, the reporter signal is used as a general label of the analytes in a sample. Each sample, however, uses a different reporter signal. This allows samples as a whole to be compared with each other. By additionally separating or distinguishing different analytes in the samples, one can easily analyze many analytes in many samples in a single assay. For example, proteins in multiple samples can be labeled with reporter signals as described above, and the samples mixed together. If some or all of the

various labeled proteins are separated by, for example, association of the proteins with antibodies on an array, the presence and amount of a given protein in each of the samples can be determined by identifying the reporter signals present at each array element. If the protein corresponding to a given array element was present in a particular sample, then some of the protein associated with that array element will be labeled with the reporter signal used to label that particular sample. Detection of that reporter signal will indicate this. This same relationship holds true for all of the other samples. Further, the amount of reporter signal detected can indicate the amount of a given protein in a given sample, and the simultaneous quantitation of protein in multiple samples can provide a particularly accurate comparison of the levels of the proteins in the various samples.

In one form of reporter signal labeling, referred to as reporter signal protein labeling (RSPL), reporter signals are used for sensitive detection of one or multiple proteins. In the method, proteins labeled with reporter signals are analyzed using the reporter signals to distinguish the labeled proteins. Detection of the reporter signals indicates the presence of the corresponding protein(s). The detected protein(s) can then be analyzed using known techniques. The labels provide a unique protein/label composition that can specifically identify the protein(s). This is accomplished through the use of the specialized reporter signals as the labels. The labeled protein(s) can be fragmented, such as by protease digestion, prior to analysis. A protein sample to be analyzed can also be subjected to fractionation or separation to reduce the complexity of the samples. Fragmentation and fractionation can also be used together in the same assay. Such fragmentation and fractionation can simplify and extend the analysis of the proteins.

C. Reporter Signal Calibration

In another form of the method, referred to as reporter signal calibration (RSC), a form of reporter signals referred to as reporter signal calibrators are mixed with analytes or analyte fragments, the reporter signal calibrators and the analytes or analyte fragments are altered, and the altered forms of the reporter signal calibrators and altered forms of the analytes or analyte fragments are detected. Reporter signal calibrators are useful as standards for assessing the amount of analytes present. That is, one can add a known amount of a reporter signal calibrator in order to assess the amount of analyte

present comparing the amount of altered analyte or analyte fragment detected with the amount of altered reporter signal calibrator detected and calibrating these amounts with the known amount of reporter signal calibrator added (and thus the predicted amount of altered reporter signal calibrator).

5 In some embodiments, each analyte or analyte fragment can share one or more common properties with at least one reporter signal calibrator such that the reporter signal calibrators and analytes or analyte fragments having the common property can be distinguished and/or separated from other molecules lacking the common property.

10 In some embodiments, reporter signal calibrators and analytes and analyte fragments can be altered such that the altered form of an analyte or analyte fragment can be distinguished from the altered form of the reporter signal calibrator with which the analyte or analyte fragment shares a common property. In some embodiments, the altered forms of different reporter signal calibrators can be distinguished from each other. In some embodiments, the altered forms of different analytes or analyte
15 fragments can be distinguished from each other.

 In some embodiments of reporter signal calibration, the analyte or analyte fragment is not altered and so the altered reporter signal calibrators and the analytes or analyte fragments are detected. In this case, the analyte or analyte fragment can be distinguished from the altered form of the reporter signal calibrator with which the
20 analyte or analyte fragment shares a common property.

 In some embodiments the analyte or analyte fragment may be a reporter signal or a fragment of a reporter signal. In this case, the reporter signal calibrators serve as calibrators for the amount of reporter signal detected.

 Reporter signal calibration is preferably used in connection with proteins and
25 peptides (as the analytes). This form of reporter signal calibration is referred to as reporter signal protein calibration. Reporter signal protein calibration is useful, for example, for producing protein signatures of protein samples. As used herein, a protein signature is the presence, absence, amount, or presence and amount of a set of proteins or protein surrogates.

30 In some embodiments of reporter signal protein calibration, the presence of labile, scissile, or cleavable bonds in the proteins to be detected can be exploited. Peptides, proteins, or protein fragments (collectively referred to, for convenience, as

protein fragments in the remaining description) containing such bonds can be altered by fragmentation at the bond. In this way, reporter signal calibrators having a common property (such as mass-to-charge ratio) with the protein fragments can be used and the altered forms of the reporter signal calibrators and the altered (that is, fragmented)

5 forms of the protein fragments can be detected and distinguished. In this regard, although the protein fragments share a common property with their matching reporter signal calibrators, the altered forms of the reporter signal calibrators and altered forms of protein fragments can be distinguished (because, for example, the altered forms have different properties, such as different mass-to-charge ratios).

10 D. Reporter Signal Fusions

In another form of the disclosed method and compositions, referred to as reporter signal fusions (RSF), reporter signal peptides are joined with a protein or peptide of interest in a single amino acid segment. Such fusions of proteins with reporter signal peptides can be expressed from a nucleic acid molecule encoding the amino acid segment that constitutes the fusion. The fusion protein is referred to herein as a reporter signal fusion. The reporter signal peptides allow for sensitive monitoring and detection of the proteins and peptides to which they are fused, and of expression of the genes, vectors, expression constructs, and nucleic acids that encode them. In particular, the reporter signal fusions allow sensitive and multiplex detection of expression of particular proteins and peptides of interest, and/or of the genes, vectors, and expression constructs encoding the proteins and peptides of interest.

The disclosed reporter signal fusions also are useful for creating cells, cell lines, and organisms that have particular protein(s), gene(s), vector(s), and/or expression sequence(s) labeled (that is, associated with or involved in) reporter signal fusions. For example, a set of nucleic acid constructs, each encoding a reporter signal fusion with a different reporter signal peptide, can be used to uniquely label a set of cells, cell lines, and/or organisms. Processing of a sample from any of the labeled sources can result in a unique altered form of the reporter signal peptide (or the amino acid segment or an amino acid subsegment) for each of the possible sources that can be distinguished from the other altered forms. Detection of a particular altered form identifies the source from which it came.

The disclosed reporter signal fusions also can be used to "label" particular pathways, regulatory cascades, and other suites of genes, proteins, vectors, and/or expressions sequences. Such labeling can be within the same cell, cell line, or organism, or across a set of cells, cell lines, or organisms. For example, nucleic acid segments encoding reporter signal fusions can be associated with endogenous expression sequences of interest, endogenous genes of interest, exogenous expression sequences of interest, exogenous genes of interest, or a combination. The exogenous constructs then are introduced into the cells or organisms of interest. The association with endogenous expression sequences or genes can be accomplished, for example, by introducing a nucleic acid molecule (encoding the reporter signal fusion) for insertion at the site of the expression sequences or gene of interest, or by creating a vector or other nucleic acid construct (containing both the endogenous expression sequences or gene and a nucleic acid segment encoding the reporter signal fusion) in vitro and introducing the construct into the cells or organisms of interest. Many other uses and modes of use are possible, a number of which are described in the illustrations below. In particular, the disclosed reporter signal fusions can be used in any context and for any purpose that green fluorescent protein and green fluorescent protein fusions are used. However, the disclosed reporter signal proteins have more uses and are more useful than green fluorescent protein at least because of the ability to multiplex the disclosed reporter signal fusions.

The reporter signal peptides can be used for sensitive detection of one or multiple proteins (that is, the proteins to which the reporter signal peptides are fused). In the method, proteins fused with reporter signal peptides are analyzed using the reporter signal peptides to distinguish the reporter signal fusions. Detection of the reporter signal peptides indicates the presence of the corresponding protein(s). The detected protein(s) can then be analyzed using known techniques. The reporter signal fusions provide a unique protein/label composition that can specifically identify the protein(s). This is accomplished through the use of the specialized reporter signal peptides as the labels.

The reporter signal fusions can be produced by expression from nucleic acid molecules encoding the fusions. Thus, the disclosed fusions generally can be designed by designing nucleic acid segments that encode amino acid segments where the amino

acid segments comprise a reporter signal peptide and a protein or peptide of interest. A given nucleic acid molecule can comprise one or more nucleic acid segments. A given nucleic acid segment can encode one or more amino acid segments. A given amino acid segment can include one or more reporter signal peptides and one or more proteins or peptides of interest. The disclosed amino acid segments consist of a single, contiguous polypeptide chain. Thus, although multiple amino acid segments can be part of the same contiguous polypeptide chain, all of the components (that is, the reporter signal peptide(s) and protein(s) and peptide(s) of interest) of a given amino acid segment are part of the same contiguous polypeptide chain.

Reporter signal peptides can be fragmented, decomposed, reacted, derivatized, or otherwise modified, preferably in a characteristic way. This allows a protein to which the reporter signal peptide is fused to be identified by detection of one or more of the products of the reporter signal fusion following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal peptide. Expression of one or more proteins in one or more samples can be detected in a multiplex manner. Preferably, for mass spectrometry reporter signals, the reporter signal peptides are fragmented to yield fragments of similar charge but different mass.

Preferably, the reporter signal peptides are fragmented to yield fragments of similar charge but different mass. This allows each reporter signal fusion (and/or each reporter signal peptide) in a set to be distinguished by the different mass-to-charge ratios of the fragments of (that is, altered forms of) the reporter signal peptides or reporter signal fusions. This is possible since the fragments of the different reporter signal peptides (or the fragments of the reporter signal fusions) can be designed to have different mass-to-charge ratios. In the disclosed method, this allows each reporter signal fusion to be distinguished by the mass-to-charge ratios of the reporter signal fusions after fragmentation of the reporter signal peptide.

Alteration of reporter signals peptides in reporter signal fusions can produce a variety of altered compositions. Any or all of these altered forms can be detected. For example, the altered form of the reporter signal peptide can be detected, the altered form of the amino acid segment (which contains the reporter signal peptide) can be detected, the altered form of a subsegment of the amino acid segment can be detected, or a combination of these can be detected. Where the reporter signal peptide is altered

by fragmentation, the result generally will be a fragment of the reporter signal peptide and an altered form of the amino acid segment containing the protein or peptide of interest and a portion of the reporter signal peptide (that is, the portion not in the reporter signal peptide fragment). The protein or peptide of interest also can be
5 fragmented. The result would be a subsegment of the amino acid segment. The amino acid subsegment would contain the reporter signal peptide and a portion of the protein or peptide of interest. When the reporter signal peptide in an amino acid subsegment is altered (which can occur before, during, or after fragmentation of the amino acid segment), the result is an altered form of the amino acid subsegment (and an altered
10 form of the reporter signal peptide). This altered form of amino acid subsegment can be detected.

As with reporter signals generally, reporter signal peptides can be used in sets where the reporter signal peptides in a set can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules
15 lacking the common property. In the case of reporter signal fusions, amino acid segments and amino acid subsegments can be used in sets where the amino acid segments and amino acid subsegments in a set can have one or more common properties that allow the amino acid segments and amino acid subsegments, respectively, to be separated or distinguished from molecules lacking the common
20 property. In general, the component(s) of the reporter signal fusions having common properties can depend on the component(s) to be detected and/or the mode of the method being used.

Nucleic acid molecules encoding reporter signal fusions can be used in sets where the reporter signal peptides in the reporter signal fusions encoded by a set of
25 nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Similarly, nucleic acid segments (which, generally, are part of nucleic acid molecules) encoding reporter signal fusions can be used in sets where the reporter
30 signal peptides in the reporter signal fusions encoded by a set of nucleic acid segments can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Other relationships between members of the sets of nucleic acid molecules, nucleic acid

segments, amino acid segments, reporter signal peptides, and proteins of interest are contemplated.

Cells, cell lines, organisms, and expression of genes and proteins can be detected using the disclosed reporter signal fusions in a variety of ways. For example, the protein and attached reporter signal peptide can be detected together, one or more peptides of the protein and the attached reporter signal peptide(s) can be detected together, the fragments of the reporter signal peptide can be detected, or a combination. Preferred detection involves detection of the reporter signal fusion both before and after fragmentation of the reporter signal peptide.

A powerful form of the disclosed method is use of reporter signal fusions to assay multiple samples (for example, time series assays or other comparative analyses). Knowledge of the temporal response of a biological system following perturbation is a very powerful process in the pursuit of understanding the system. To follow the temporal response a sample of the system is obtained (for example, cells from a cell culture, mice initially synchronized and sacrificed) at determined times following the perturbation. Knowledge of spatial protein profiles (for example, relative position within a tissue section) is a very powerful process in the pursuit of understanding the biological system.

The reporter signal fusions are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge ratios. A set of isobaric reporter signal peptides or reporter signal fusions can be used for multiplex labeling and/or detection of the expression of many genes, proteins, vectors, expression constructs, cells, cell lines, and organisms since the reporter signal peptide fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection. Where the same gene, protein, vector, expression construct, cell, cell line, or organism (or the same type of gene, protein, vector, expression construct, cell, cell line, or organism) is labeled with a set of reporter signal fusions that are isobaric or that include isobaric reporter signal peptides (by, for example, "labeling" the same gene, protein, vector, expression construct, cell, cell line, or organism in different samples), the set of reporter signal fusions or reporter signal peptides that results will also be isobaric. Fragmentation of the reporter signal peptides will split the set of reporter signal peptides into individually detectable reporter signal

fusion fragments and reporter signal peptide fragments of characteristically different mass.

5 A preferred form of the disclosed method involves filtering of isobaric reporter signal fusions or reporter signal peptides from other molecules based on mass-to-charge ratio, fragmentation of the reporter signal peptides to produce fragments having different masses, and detection of the different fragments based on their mass-to-charge ratios. The method is best carried out using a tandem mass spectrometer as described elsewhere herein.

10 It is an object of the present invention to provide a method for the multiplexed determination of presence, amount, or presence and amount of analytes.

It is an object of the present invention to provide labeled proteins such that the presence, amount, or presence and amount of the proteins can be determined.

15 It is another object of the present invention to provide a method for labeling proteins so as to allow the multiplexed determination of presence, amount, or presence and amount of proteins.

It is another object of the present invention to provide a method for the multiplexed determination of presence, amount, or presence and amount of proteins.

It is an object of the present invention to provide a method for detecting a mass tag signature.

20 It is an object of the present invention to provide a method for detecting a protein signature.

It is another object of the present invention to provide an assessment of the identity and purity of the peptides comprising a protein signature.

25 It is another object of the present invention to provide a method for detecting phosphopeptides, or other posttranslational protein modifications, among the peptides comprising a protein signature.

It is another object of the present invention to provide kits for generating mass tag signatures.

30 It is another object of the present invention to provide kits for generating protein signatures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are graphs of mass-to-charge ratio (m/z) versus signal intensity. Figure 1A shows the results where there is no fragmentation of the reporter signal. A single peak represents the parent ion. Figure 1B shows the results where the reporter signal is fragmented. The parent ion along with two fragmentation ions are detected.

Figures 2A and 2B are graphs of mass-to-charge ratio (m/z) versus detected counts. Figure 2A shows the results where no fragmentation of reporter signals A and B occurs. Figure 2B shows the results where all of the reporter signals are fragmented (A fragments to A1 and A2, B fragments to B1 and B2).

Figure 3 is an example of an ESI-TOF mass spectrum of an example of a reporter signal peptide (LAT3838 in this case). Most of the complexity of the spectrum comes from fragmentation of the reporter signal peptide in the source.

Figure 4 is an example a spectrum of a selected reporter signal peptide (LAT3838 in this case) following fragmentation. The parent reporter signal was selected at a filter setting of $m/z = 1044$ and altered by fragmented by collision with argon gas at about 20 eV collision energy. The daughter reporter signal peptide fragment at $m/z = 600$ corresponds to the expected PAGSLR⁺ fragment (amino acids 6-11 of SEQ ID NO:2).

Figure 5 is an example of a spectrum of the fragmentation products of five reporter signal peptides (LAT3838 and LAT3843 through LAT3856). The peaks corresponding to the reporter signal peptide fragments of each are labeled.

Figure 6 is an example of a spectrum showing the effect of the loss of a phosphate group from reporter signal peptide fragments.

Figure 7 is an example of a spectrum showing differentiation of reporter signal peptide fragments based on the use of stable isotopes in the reporter signal peptides.

Figure 8 is an example of a spectrum of a complex mixture of molecules that includes five reporter signal peptides ($m/z = 1389.6$).

Figures 9A and 9B are an example of a spectrum of a set of reporter signal peptides following selection based on mass-to-charge ratio (m/z around 1390) and illustrating a nearly non-existent background everywhere except at m/z around 1390. This is the same sample (prior to selection) from which the complex spectrum of Figure

8 was obtained. Figure 9B is an expanded view of the peak. Selection was not perfect in this example as the finite resolution of the filter allowed three peaks to pass.

Figure 10 is an example of a spectrum of the fragmentation products of the selected reporter signal peptides of Figure 9 (originally in the complex sample of Figure 8). Five prominent peaks of approximately the same magnitude appear at the expected m/z of the reporter signal peptide fragments of each of the five reporter signal peptides. Unfragmented reporter signal peptides remain in the rightmost peak (m/z near 1390).

DETAILED DESCRIPTION OF THE INVENTION

Disclosed are compositions and methods for sensitive detection of one or multiple analytes. In general, the methods involve the use of special label components, referred to as reporter signals, that can be associated with, incorporated into, or otherwise linked to the analytes. Reporter signals can also be used merely in conjunction with analytes, with no significant association between the analytes and reporter signals. Compositions where reporter signals are associated with, incorporated into, or otherwise linked to the analytes are referred to as reporter signal/analyte conjugates. Such conjugates include reporter signals associated with analytes, such as a reporter signal probe hybridized to a nucleic acid sequence; reporter signals covalently coupled to analytes, such as reporter signals linked to proteins via a linking group; and reporter signals incorporated into analytes, such as fusions between a protein of interest and a peptide reporter signal.

In some embodiments, the reporter signals can be altered such that the altered forms of different reporter signals can be distinguished from each other. Reporter signal/analyte conjugates can be altered, generally through alteration of the reporter signal portion of the conjugate, such that the altered forms of different reporter signals, altered forms of different reporter signal/analyte conjugates, or both, can be distinguished from each other. Where the reporter signal or reporter signal/analyte conjugate is altered by fragmentation, any, some, or all of the fragments can be distinguished from each other, depending on the embodiment. For example, where reporter signals fragmented into two parts, either or both parts of the reporter signals can be distinguished. Where reporter signal/analyte conjugates are fragmented into two parts (with the break point in the reporter signal portion), either the reporter signal

fragment, the reporter signal/analyte fragment, or both can be distinguished. In some embodiments, only one part of a fragmented reporter signal will be detected and so only this part of the reported signals need be distinguished.

5 In some embodiments, sets of reporter signals can be used where two or more of the reporter signals in a set have one or more common properties that allow the reporter signals having the common property to be distinguished and/or separated from other molecules lacking the common property. In other embodiments, sets of reporter signal/analyte conjugates can be used where two or more of the reporter signal/analyte conjugates in a set have one or more common properties that allow the reporter
10 signal/analyte conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. In still other embodiments, analytes can be fragmented (prior to or following conjugation) to produce reporter signal/analyte fragment conjugates (which can be referred to as fragment conjugates). In such cases, sets of fragment conjugates can be used where
15 two or more of the fragment conjugates in a set have one or more common properties that allow the fragment conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. It should be understood that fragmented analytes can be considered analytes in their own right. In this light, reference to fragmented analytes is made for convenience and clarity in
20 describing certain embodiments and to allow reference to both the base analyte and the fragmented analyte.

As indicated above, reporter signals conjugated with analytes can be altered while in the conjugate and distinguished. Conjugated reporter signals can also be dissociated or separated, in whole or in part, from the conjugated analytes prior to their
25 alteration. Where the reporter signals are dissociated (in whole or in part) from the analytes, the method can be performed such that the fact of association between the analyte and reporter signal is part of the information obtained when the reporter signal is detected. In other words, the fact that the reporter signal may be dissociated from the analyte for detection does not obscure the information that the detected reporter signal
30 was associated with the analyte.

Reporter signals can also be in conjunction with analytes (such as in mixtures of reporter signals and analytes), where no significant physical association between the

reporter signals and analytes occurs; or alone, where no analyte is present. In such cases, where reporter signals are not or are no longer associated with analytes, sets of reporter signals can be used where two or more of the reporter signals in a set have one or more common properties that allow the reporter signals having the common property to be distinguished and/or separated from other molecules lacking the common property.

Detection of the reporter signals indicates the presence of the corresponding analytes. The reporter signals can have two key features. First, the reporter signals can be used in sets where all the reporter signals in the set have similar properties. The similar properties allow the reporter signals to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signals in a set have the same mass-to-charge ratio (m/z). That is, the reporter signals in a set are isobaric. This allows the reporter signals to be separated precisely from other molecules based on mass-to-charge ratio. The result of the filtering is a huge increase in the signal to noise ratio (S/N) for the system, allowing more sensitive and accurate detection.

Second, all the reporter signals in a set can be fragmented, decomposed, reacted, derivatized, or otherwise modified to distinguish the different reporter signals in the set. For example, the reporter signals can be fragmented to yield fragments having the same charge but different mass. This allows each reporter signal in a set to be distinguished by the different mass-to-charge ratios of the fragments of the reporter signals. This is possible since, although the unfragmented reporter signals in a set are isobaric, the fragments of the different reporter signals are not. Reporter signals to be detected on the basis of mass-to-charge ratio and/or to be detected with the use of a mass spectrometer, can be referred to as mass spectrometer reporter signals.

Differential distribution of mass in the fragments of the reporter signals can be accomplished in a number of ways. For example, reporter signals of the same nominal structure (for example, peptides having the same amino acid sequence), can be made with different distributions of heavy isotopes, such as deuterium. All reporter signals in the set would have the same number of a given heavy isotope, but the distribution of these would differ for different reporter signals. Similarly, reporter signals of the same general structure (for example, peptides having the same amino acid sequence), can be

made with different distributions of modifications, such as methylation, phosphorylation, sulphation, and use of seleno-methionine for methionine. All reporter signals in the set would have the same number of a given modification, but the distribution of these would differ for different reporter signals. Reporter signals of the same nominal composition (for example, made up of the same amino acids), can be made with different ordering of the subunits or components of the reporter signal. All reporter signals in the set would have the same number of subunits or components, but the distribution of these would be different for different reporter signals. Reporter signals having the same nominal composition (for example, made up of the same amino acids), can be made with a labile or scissile bond at a different location in the reporter signal. All reporter signals in the set would have the same number and order of subunits or components. Where the labile or scissile bond is present between particular subunits or components, the order of subunits or components in the reporter signal can be the same except for the subunits or components creating the labile or scissile bond. Each of these modes can be combined with one or more of the other modes to produce differential distribution of mass in the fragments of the reporter signals. For example, different distributions of heavy isotopes can be used in reporter signals where a labile or scissile bond is placed in different locations.

The reporter signals are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge ratios. The disclosed reporter signals can be used as general labels in myriad labeling and/or detection techniques. A set of isobaric reporter signals can be used for multiplex labeling and/or detection of many analytes since the reporter signal fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection.

Current technologies are limited in their ability to multiplex labels. In contrast, the disclosed method allows the readout of many samples simultaneously and high internal accuracy in comparison to a sequential readout system.

A preferred form of the disclosed method involves filtering of isobaric reporter signals from other molecules based on mass-to-charge ratio, fragmentation of the reporter signals to produce fragments having different masses, and detection of the different fragments based on their mass-to-charge ratios. The method is best carried

out using a tandem mass spectrometer where the isobaric reporter signals are passed through a filtering quadrupole, the reporter signals are fragmented in a collision cell, and the fragments are distinguished and detected in a time-of-flight (TOF) stage. In such an instrument the sample is ionized in the source (for example, in a MALDI ion source) to produce charged ions. It is preferred that the ionization conditions are such that primarily a singly charged parent ion is produced. A first quadrupole, Q0, is operated in radio frequency (RF) mode only and acts as an ion guide for all charged particles. The second quadrupole, Q1, is operated in RF + DC mode to pass only a narrow range of mass-to-charge ratios (that includes the mass-to-charge ratio of the reporter signals). This quadrupole selects the mass-to-charge ratio of interest. Quadrupole Q2, surrounded by a collision cell, is operated in RF only mode and acts as ion guide. The collision cell surrounding Q2 will be filled to appropriate pressure with a gas to fracture the input ions by collisionally induced dissociation. The collision gas preferably is chemically inert, but reactive gases can also be used. Preferred molecular systems utilize reporter signals that contain scissile bonds, labile bonds, or combinations, such that these bonds will be preferentially fractured in the Q2 collision cell.

A. Reporter Molecule Labeling

In one form of the disclosed method, referred to as reporter molecule labeling (RML), reporter signals are associated with analytes to be detected and/or quantitated. For example, a reporter signal can be associated with a specific binding molecule that interacts with the analyte of interest. Such a combination is referred to as a reporter molecule. The specific binding molecule in the reporter molecule interacts with the analyte thus associating the reporter signal with the analyte. Alternatively, a reporter signal can be associated with an analyte indirectly. In this mode, a "coding" molecule containing a specific binding molecule and a coding tag is associated with the analyte (via the specific binding molecule). Alternatively, a coding tag can be coupled or directly associated with the analyte. Then a reporter signal associated with a decoding tag (such a combination is another form of reporter molecule) is associated with the coding molecule through an interaction between the coding tag and the decoding tag. An example of this interaction is hybridization where the coding and decoding tags are complementary nucleic acid sequences. The result is an indirect association of the

reporter signal with the analyte. This mode has the advantage that all of the interactions of the reporter signals with the coding molecule can be made chemically and physically similar by using the same types of coding tags and decoding tags for all of the coding molecules and reporter molecules in a set.

5 **B. Reporter Signal Labeling**

10 In another form of the disclosed method, referred to as reporter signal labeling (RSL), reporter signals are used for sensitive detection of one or multiple analytes. In the method, analytes labeled with reporter signals are analyzed using the reporter signals to distinguish the labeled analytes (where the analytes are labeled with the reporter signals). Detection of the reporter signals indicates the presence of the corresponding analyte(s). The detected analyte(s) can then be analyzed using known techniques. The labels provide a unique analyte/label composition that can specifically identify the analyte(s). This is accomplished through the use of the reporter signals as the labels. The labeled analyte(s) can be fragmented prior to analysis. An analyte
15 sample to be analyzed can also be subjected to fractionation or separation to reduce the complexity of the samples. Fragmentation and fractionation can also be used together in the same assay. Such fragmentation and fractionation can simplify and extend the analysis of the analytes.

20 Reporter signals can be coupled or directly associated with an analyte. For example, a reporter signal can be coupled to an analyte via reactive groups, or a reporter molecule (composed of a specific binding molecule and a reporter signal) can be associated with an analyte. The reporter signals can be attached to analytes in any manner. For example, reporter signals can be covalently coupled to proteins through a sulfur-sulfur bond between a cysteine on the protein and a cysteine on the reporter
25 signal. Many other chemistries and techniques for coupling compounds to analytes are known and can be used to couple reporter signals to analytes. For example, coupling can be made using thiols, epoxides, nitriles for thiols, NHS esters, isothiocyanates for amines, and alcohols for carboxylic acids. Reporter signals can be attached to analytes either directly or indirectly, for example, via a linker.

30 Reporter signals, or constructs containing reporters signals, also can be attached or coupled to analytes by ligation. Methods for ligation of nucleic acids are well known (see, for example, Sambrook et al. Molecular Cloning: A Laboratory Manual,

second edition, 1989, Cold Spring Harbor Laboratory Press, New York.), and efficient protein ligation is known (see, for example, Dawson et al., "Synthesis of proteins by native chemical ligation" Science 266, 776-9 (1994); Hackeng et al., "Chemical synthesis and spontaneous folding of a multidomain protein: anticoagulant microprotein S" Proc Natl Acad Sci USA 97:14074-8 (2000); Dawson et al., "Synthesis of Native Proteins by Chemical Ligation" Ann. Rev. Biochem. 69:923-960 (2000); U.S. Patent No. 6,184,344; PCT Publication WO 98/28434).

Alternatively, a reporter signal can be associated with an analyte indirectly. In this mode, a "coding" molecule containing a specific binding molecule and a coding tag can be associated with the analyte (via the specific binding molecule). Alternatively, a coding tag can be coupled or directly associated with the analyte. Then a reporter signal associated with a decoding tag (such a combination is another form of reporter molecule) is associated with the coding molecule through an interaction between the coding tag and the decoding tag. An example of this interaction is hybridization where the coding and decoding tags are complementary nucleic acid sequences. The result is an indirect association of the reporter signal with the analyte. This mode has the advantage that all of the interactions of the reporter signals with the coding molecule can be made chemically and physically similar by using the same types of coding tags and decoding tags for all of the coding molecules and reporter molecules in a set.

Reporter signals can be fragmented, decomposed, reacted, derivatized, or otherwise modified, preferably in a characteristic way. This allows an analyte to which the reporter signal is attached to be identified by the correlated detection of the labeled analyte and one or more of the products of the labeled analyte following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal (the labeled analyte is the analyte/reporter signal combination). The alteration of the reporter signal will alter the labeled analyte in a characteristic and detectable way. Together, the detection of a characteristic labeled analyte and a characteristic product of the labeled analyte can uniquely identify the analyte. In this way, using the disclosed method and materials, one or more analytes can be detected, either alone or together (for example, in a multiplex assay). Further, one or more analytes in one or more samples can be detected in a multiplex manner. Preferably, for mass

spectrometry reporter signals, the reporter signals are fragmented to yield fragments of similar charge but different mass.

Preferably, the reporter signals are fragmented to yield fragments of similar charge but different mass. This allows each labeled analyte (and/or each reporter signal) in a set to be distinguished by the different mass-to-charge ratios of the fragments of the reporter signals. This is possible since, although the unfragmented reporter signals in a set are isobaric, the fragments of the different reporter signals are not. In the disclosed method, this allows each analyte/reporter signal combination to be distinguished by the mass-to-charge ratios of the analyte/reporter signals after fragmentation of the reporter signal.

In preferred embodiments, reporter signals are used in sets where all the reporter signals in the set have similar properties (such as similar mass-to-charge ratios). The similar properties allow the reporter signals to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signals in a set have the same mass-to-charge ratio (m/z). That is, the reporter signals in a set are isobaric. This allows the reporter signals (or any analytes to which they are attached) to be separated precisely from other molecules based on mass-to-charge ratio. The result of the filtering is a huge increase in the signal to noise ratio (S/N) for the system, allowing more sensitive and accurate detection. Alternatively, or in addition, reporter signals can be used in sets such that the resulting labeled analytes will have similar properties allowing the labeled analytes to be distinguished and/or separated from other molecules lacking one or more of the properties.

Analytes can be detected using the disclosed reporter signals in a variety of ways. For example, the analyte and attached reporter signal can be detected together, one or more fragments of the analyte and the attached reporter signal(s) can be detected together, the fragments of the reporter signal can be detected, or a combination. Preferred detection involves detection of the analyte/reporter signal both before and after fragmentation of the reporter signal.

A preferred form of the disclosed method involves correlated detection of the reporter signals both before and after fragmentation of the reporter signal. This allows labeled analytes to be detected and identified via the change in labeled analyte. That is, the nature of the reporter signal detected (non-fragmented versus fragmented) identifies

the analyte as labeled. Where the analytes and reporter signals are detected by mass-to-charge ratio, the change in mass-to-charge ratio between fragmented and non-fragmented samples provides the basis for comparison. Such mass-to-charge ratio detection is preferably accomplished with mass spectrometry.

5 As an example, an analyte in a sample can be labeled with reporter signal designed as a mass spectrometry label. The labeled analyte can be subjected to mass spectrometry. A peak corresponding to the analyte/reporter signal will be detected. Fragmentation of the reporter signal in the mass spectrometer (preferably in a collision cell) would result in a shift in the peak corresponding to the loss of a portion of the
10 attached reporter signal, the appearance of a peak corresponding to the lost fragment, or a combination of both events. Significantly, the shift observed will depend on which reporter signal is on the analyte since different reporter signals will, by design, produce fragments with different mass-to-charge ratios. The combination event of detection of the parent mass-to-charge (with no collision gas) and the mass-to-charge corresponding
15 to the loss of the fragment from the reporter signal (with collision gas) indicates a labeled analyte. The identity of the analyte can be determined by standard mass spectrometry techniques, such as compositional analysis.

A powerful form of the disclosed method is use of analytes labeled with reporter signals to assay multiple samples (for example, time series assays or other
20 comparative analyses). Knowledge of the temporal response of a biological system following perturbation is a very powerful process in the pursuit of understanding the system. To follow the temporal response, a sample of the system is obtained (for example, cells from a cell culture, mice initially synchronized and sacrificed) at determined times following the perturbation. Knowledge of spatial analyte profiles (for
25 example, relative position within a tissue section) is a very powerful process in the pursuit of understanding the biological system.

In the disclosed method a series of samples can each be labeled with a different reporter signal from a set of reporter signals. Preferred reporter signals for this purpose would be those using differentially distributed mass. In particular, the use of stable
30 isotopes is preferred to ensure that members of the set of reporter signals would behave chemically identically and yet would be distinguishable.

The labeled analytes are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge ratios. The disclosed reporter signals can be used as general labels in myriad labeling and/or detection techniques. A set of isobaric reporter signals can be used for multiplex
5 labeling and/or detection of many analytes since the reporter signal fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection. Where the same analyte or type of analyte is labeled with a set of isobaric reporter signals (by, for example, labeling the same analyte in different samples), the set of labeled analytes that results from use of an isobaric set of reporter
10 signals will also be isobaric. Fragmentation of the reporter signals will split the set of labeled analytes into individually detectable labeled proteins of characteristically different mass.

A preferred form of the disclosed method involves filtering of isobaric reporter signals (and the attached analytes) from other molecules based on mass-to-charge ratio,
15 fragmentation of the reporter signals to produce fragments having different masses, and detection of the different fragments based on their mass-to-charge ratios. This form of the method is best carried out using a tandem mass spectrometer as described elsewhere herein.

The disclosed method can be used in many modes. For example, the disclosed
20 method can be used to detect a specific analyte (in a specific sample or in multiple samples) or multiple analytes (in a single sample or multiple samples). In each case, the analyte(s) to be detected can be separated either from other, unlabeled analytes or from other molecules lacking a property of the labeled analyte(s) to be detected. For example, analytes in a sample can be generally labeled with reporter signals and some
25 analytes can be separated on the basis of some property of the analytes. For example, the separated analytes could have a certain mass-to-charge ratio (separation based on mass-to-charge ratio will select both labeled and unlabeled analytes having the selected mass-to-charge ratio). As another example, all of the labeled analytes can be distinguished and/or separated from unlabeled molecules based on a feature of the
30 reporter signal such as an affinity tag. Where different affinity tags are used, some labeled analytes can be distinguished and/or separated from others. Reporter signal labeling allows profiling of analytes and cataloging of analytes.

In one mode of the disclosed method, multiple analytes in multiple samples are labeled where all of the analytes in a given sample are labeled with the same reporter signal. That is, the reporter signal is used as a general label of the analytes in a sample. Each sample, however, uses a different reporter signal. This allows samples as a whole to be compared with each other. By additionally separating or distinguishing different analytes in the samples, one can easily analyze many analytes in many samples in a single assay. For example, proteins in multiple samples can be labeled with reporter signals as described above, and the samples mixed together. If some or all of the various labeled proteins are separated by, for example, association of the proteins with antibodies on an array, the presence and amount of a given protein in each of the samples can be determined by identifying the reporter signals present at each array element. If the protein corresponding to a given array element was present in a particular sample, then some of the protein associated with that array element will be labeled with the reporter signal used to label that particular sample. Detection of that reporter signal will indicate this. This same relationship holds true for all of the other samples. Further, the amount of reporter signal detected can indicate the amount of a given protein in a given sample, and the simultaneous quantitation of protein in multiple samples can provide a particularly accurate comparison of the levels of the proteins in the various samples.

1. Reporter Signal Protein Labeling

In one form of reporter signal labeling, referred to as reporter signal protein labeling (RSPL), reporter signals are used for sensitive detection of one or multiple proteins. In the method, proteins labeled with reporter signals are analyzed using the reporter signals to distinguish the labeled proteins. Detection of the reporter signals indicates the presence of the corresponding protein(s). The detected protein(s) can then be analyzed using known techniques. The labels provide a unique protein/label composition that can specifically identify the protein(s). This is accomplished through the use of the specialized reporter signals as the labels.

Although reference is made above and elsewhere herein to detection of a "protein" or "proteins," the disclosed method and compositions encompass proteins, peptides, and fragments of proteins or peptides. Thus, reference to a protein herein is intended to refer to proteins, peptides, and fragments of proteins or peptides unless the

context clearly indicates otherwise. As used herein "labeled protein" refers to a protein, peptide, or fragment of a protein or peptide to which a reporter signal is attached unless the context clearly indicates otherwise. The labeled protein(s) can be fragmented, such as by protease digestion, prior to analysis. A protein sample to be analyzed can also be
5 subjected to fractionation or separation to reduce the complexity of the samples. Fragmentation and fractionation can also be used together in the same assay. Such fragmentation and fractionation can simplify and extend the analysis of the proteins.

The reporter signals can be attached to proteins in any manner. For example, reporter signals can be covalently coupled to proteins through a sulfur-sulfur bond
10 between a cysteine on the protein and a cysteine on the reporter signal. Many other chemistries and techniques for coupling compounds to proteins are known and can be used to couple reporter signals to proteins. For example, coupling can be made using thiols, epoxides, nitriles for thiols, NHS esters, isothiocyanates for amines, and alcohols for carboxylic acids. Reporter signals can be attached to proteins either directly or
15 indirectly, for example, via a linker. Reporter signals also can be attached to proteins by ligation (for example, protein ligation of a reporter signal peptide to a protein).

It is possible to form labeled proteins where the reporter signal is specifically attached to phosphopeptides. Chemistry for specific derivatization of phosphoserine or phosphotyrosine residues has been described (Zhou *et al.* *A systematic approach to the
20 problem of protein phosphorylation.*, Nat. Biotech. 19:375-378 (2001), Oda *et al.*, *Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome.*, Nat. Biotech. 19:379-382 (2001)). Tryptic peptides treated according to either of these two protocols will display reactive sulfhydryls at sites of protein phosphorylation. These sites may be reacted with reporter signals to generate a
25 labeled protein. Non-phosphorylated peptides will not be derivatized.

Reporter signals can be fragmented, decomposed, reacted, derivatized, or otherwise modified, preferably in a characteristic way. This allows a protein to which the reporter signal is attached to be identified by the correlated detection of the labeled protein and one or more of the products of the labeled protein following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal (the
30 labeled protein is the protein/reporter signal combination). The alteration of the reporter signal will alter the labeled protein in a characteristic and detectable way.

Together, the detection of a characteristic labeled protein and a characteristic product of the labeled protein can uniquely identify the protein. In this way, using the disclosed method and materials, one or more proteins can be detected, either alone or together (for example, in a multiplex assay). Further, one or more proteins in one or more samples can be detected in a multiplex manner. Preferably, for mass spectrometry reporter signals, the reporter signals are fragmented to yield fragments of similar charge but different mass.

Preferably, the reporter signals are fragmented to yield fragments of similar charge but different mass. This allows each labeled protein (and/or each reporter signal) in a set to be distinguished by the different mass-to-charge ratios of the fragments of the reporter signals. This is possible since, although the unfragmented reporter signals in a set are isobaric, the fragments of the different reporter signals are not. In the disclosed method, this allows each protein/reporter signal combination to be distinguished by the mass-to-charge ratios of the protein/reporter signals after fragmentation of the reporter signal.

In preferred embodiments, reporter signals are used in sets where all the reporter signals in the set have similar properties (such as similar mass-to-charge ratios). The similar properties allow the reporter signals to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signals in a set have the same mass-to-charge ratio (m/z). That is, the reporter signals in a set are isobaric. This allows the reporter signals (or any proteins to which they are attached) to be separated precisely from other molecules based on mass-to-charge ratio. The result of the filtering is a huge increase in the signal to noise ratio (S/N) for the system, allowing more sensitive and accurate detection. Alternatively, or in addition, reporter signals can be used in sets such that the resulting labeled proteins will have similar properties allowing the labeled proteins to be distinguished and/or separated from other molecules lacking one or more of the properties.

Proteins can be detected using the disclosed reporter signals in a variety of ways. For example, the protein and attached reporter signal can be detected together, one or more peptides of the protein and the attached reporter signal(s) can be detected together, the fragments of the reporter signal can be detected, or a combination.

Preferred detection involves detection of the protein/reporter signal or peptide/reporter signal both before and after fragmentation of the reporter signal.

A preferred form of the disclosed method involves correlated detection of the reporter signals both before and after fragmentation of the reporter signal. This allows
5 labeled proteins to be detected and identified via the change in labeled protein. That is, the nature of the reporter signal detected (non-fragmented versus fragmented) identifies the protein as labeled. Where the proteins and reporter signals are detected by mass-to-charge ratio, the change in mass-to-charge ratio between fragmented and non-fragmented samples provides the basis for comparison. Such mass-to-charge ratio
10 detection is preferably accomplished with mass spectrometry.

As an example, a protein in a sample can be labeled with reporter signal designed as a mass spectrometry label. The labeled protein can be subjected to tryptic digest followed by mass spectrometry of the resulting materials. A peak corresponding to the tryptic fragment containing the reporter signal will be detected. Fragmentation of
15 the reporter signal in the mass spectrometer (preferably in a collision cell) would result in a shift in the peak corresponding to the loss of a portion of the attached reporter signal, the appearance of a peak corresponding to the lost fragment, or a combination of both events. Significantly, the shift observed will depend on which reporter signal is on the protein since different reporter signals will, by design, produce fragments with
20 different mass-to-charge ratios. The combination event of detection of the parent mass-to-charge (with no collision gas) and the mass-to-charge corresponding to the loss of the fragment from the reporter signal (with collision gas) indicates a labeled protein. The combination event may be carried out in an analogous fashion to the detection of phosphorylation sites described above. The identity of the tryptic fragment of the
25 protein can be determined by standard mass spectrometry techniques, such as compositional analysis and peptide sequencing.

Not all labeled protein fragments that can be made in the disclosed method from a protein sample will be unique. Because some proteins have common motifs that may be identical in different proteins, some protein fragments or peptides produced from a
30 sample will be identical although they were derived from different proteins. For example, some families of related proteins have such common motifs or common amino acid sequences. Thus, in some embodiments of the disclosed method, detection

of a characteristic labeled protein may be the result of detection of a common portion of related proteins. Such a result can be an advantage when detection of the family of proteins is desired. Alternatively, such collective detection of related proteins can be avoided by focusing on detection of unique fragments (that is, non-identical portions) of the proteins in the family. For convenience, as used herein, detection of a common portion of multiple related proteins is intended to be encompassed by reference to detection of a unique protein, labeled protein, or other component, unless the context clearly indicates otherwise.

A powerful form of the disclosed method is use of proteins labeled with reporter signals to assay multiple samples (for example, time series assays or other comparative analyses). Knowledge of the temporal response of a biological system following perturbation is a very powerful process in the pursuit of understanding the system. To follow the temporal response a sample of the system is obtained (for example, cells from a cell culture, mice initially synchronized and sacrificed) at determined times following the perturbation. Knowledge of spatial protein profiles (for example, relative position within a tissue section) is a very powerful process in the pursuit of understanding the biological system.

In the disclosed method a series of samples can each be labeled with a different reporter signal from a set of reporter signals. Preferred reporter signals for this purpose would be those using differentially distributed mass. In particular, the use of stable isotopes is preferred to ensure that members of the set of reporter signals would behave chemically identically and yet would be distinguishable. An exemplary set of labels could be as shown in Table 1, where each of five time points could be labeled with one of the five indicated labels and the mixture of the samples could be read out simultaneously. The unfragmented labels are SEQ ID NO:1 and the fragmented labels are amino acids 7-12 of SEQ ID NO:1.

Table 1

Sequence	Mass (amu)	Fragment Sequence	Fragment mass (amu)
CG*G*G*G*DPGGGGR	949	PGGGGR	499
CG*G*G*GDPGGGG*R	949	PGGGG*R	500
CG*G*GGDPGGG*G*R	949	PGGG*G*R	501
CG*GGGDPGG*G*G*R	949	PGG*G*G*R	502
CGGGGDPG*G*G*G*R	949	PG*G*G*G*R	503

The labeled proteins are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge ratios.

5 The disclosed reporter signals can be used as general labels in myriad labeling and/or detection techniques. A set of isobaric reporter signals can be used for multiplex labeling and/or detection of many proteins since the reporter signal fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection. Where the same protein or type of protein is labeled with a set of
10 isobaric reporter signals (by, for example, labeling the same protein in different samples), the set of labeled proteins that results from use of an isobaric set of reporter signals will also be isobaric. Fragmentation of the reporter signals will split the set of labeled proteins into individually detectable labeled proteins of characteristically different mass.

15 A preferred form of the disclosed method involves filtering of isobaric reporter signals (and the attached proteins) from other molecules based on mass-to-charge ratio, fragmentation of the reporter signals to produce fragments having different masses, and detection of the different fragments based on their mass-to-charge ratios. The method is best carried out using a tandem mass spectrometer as described elsewhere herein.

20 The method allows detection of proteins, peptides and protein fragments where detection provides some information on the sequence or other structure of the protein or peptide detected. For example, the mass or mass-to-charge ratio, the amino acid composition, or amino acid sequence can be determined. The set of proteins, peptides and/or protein fragments detected in a sample using particular reporter signals will

produce characteristic sets of protein and peptide information. The method allows a complex sample of proteins to be cataloged quickly and easily in a reproducible manner. The disclosed method also should produce two "signals" for each protein, peptide, or peptide fragment in the sample: the original labeled protein and the altered form of the labeled protein. This can allow comparisons and validation of a set of detected proteins and peptides.

Reporter signal protein labeling allows profiling of proteins, de novo discovery of proteins, and cataloging of proteins. The method has advantageous properties which can be used as a detection and analysis system for protein analysis, proteome analysis, proteomic, protein expression profiling, de novo protein discovery, functional genomics, and protein detection.

C. Reporter Signal Calibration

In another form of the method, referred to as reporter signal calibration (RSC), a form of reporter signals referred to as reporter signal calibrators are mixed with analytes or analyte fragments, the reporter signal calibrators and the analytes or analyte fragments are altered, and the altered forms of the reporter signal calibrators and altered forms of the analytes or analyte fragments are detected. Reporter signal calibrators are useful as standards for assessing the amount of analytes present. That is, one can add a known amount of a reporter signal calibrator in order to assess the amount of analyte present comparing the amount of altered analyte or analyte fragment detected with the amount of altered reporter signal calibrator detected and calibrating these amounts with the known amount of reporter signal calibrator added (and thus the predicted amount of altered reporter signal calibrator).

The disclosed reporter signal calibration method generates, with high sensitivity, unique protein signatures related to the relative abundance of different proteins in tissue, microorganisms, or any other biological sample. The disclosed method allows one to define the status of a cell or tissue by identifying and measuring the relative concentrations of a small but highly informative subset of proteins. Such a measurement is known as a protein signature. Protein signatures are useful, for example, in the diagnosis, grading, and staging of cancer, in drug screening, and in toxicity testing.

In some embodiments, each analyte or analyte fragment can share one or more common properties with at least one reporter signal calibrator such that the reporter signal calibrators and analytes or analyte fragments having the common property can be distinguished and/or separated from other molecules lacking the common property.

5 In some embodiments, reporter signal calibrators and analytes and analyte fragments can be altered such that the altered form of an analyte or analyte fragment can be distinguished from the altered form of the reporter signal calibrator with which the analyte or analyte fragment shares a common property. In some embodiments, the altered forms of different reporter signal calibrators can be distinguished from each
10 other. In some embodiments, the altered forms of different analytes or analyte fragments can be distinguished from each other.

In some embodiments of reporter signal calibration, the analyte or analyte fragment is not altered and so the altered reporter signal calibrators and the analytes or analyte fragments are detected. In this case, the analyte or analyte fragment can be
15 distinguished from the altered form of the reporter signal calibrator with which the analyte or analyte fragment shares a common property.

In some embodiments the analyte or analyte fragment may be a reporter signal or a fragment of a reporter signal. In this case, the reporter signal calibrators serve as calibrators for the amount of reporter signal detected.

20 Reporter signal calibration is preferably used in connection with proteins and peptides (as the analytes). This form of reporter signal calibration is referred to as reporter signal protein calibration. Reporter signal protein calibration is useful, for example, for producing protein signatures of protein samples. As used herein, a protein signature is the presence, absence, amount, or presence and amount of a set of proteins
25 or protein surrogates.

In some embodiments of reporter signal protein calibration, the presence of labile, scissile, or cleavable bonds in the proteins to be detected can be exploited. Peptides, proteins, or protein fragments (collectively referred to, for convenience, as protein fragments in the remaining description) containing such bonds can be altered by
30 fragmentation at the bond. In this way, reporter signal calibrators having a common property (such as mass-to-charge ratio) with the protein fragments can be used and the altered forms of the reporter signal calibrators and the altered (that is, fragmented)

forms of the protein fragments can be detected and distinguished. In this regard, although the protein fragments share a common property with their matching reporter signal calibrators, the altered forms of the reporter signal calibrators and altered forms of protein fragments can be distinguished (because, for example, the altered forms have
5 different properties, such as different mass-to-charge ratios).

As an example of reporter signal protein calibration, a protein sample of interest can be digested with a serine protease, preferably trypsin. The digest generates a complex mixture of protein fragments. Among these protein fragments, there will exist a subset (approximately one protein fragment among every 400) that contains the
10 dipeptide Asp-Pro. This dipeptide sequence is uniquely sensitive to fragmentation during mass spectrometry, and thus produces a high yield of ions in fragmentation mode. Since the human proteome consists of at least 2,000,000 distinct tryptic peptides, the number of protein fragments containing the Asp-Pro sequence is of the order of 5,000. Since some of these may exist as phosphopeptides or other modified forms, the number
15 may be even higher. This number is sufficiently high to permit the selection of a subset (perhaps 50 to 100 or so) of fragmentable protein fragments that is suitable for generating a highly informative protein signature. Peptides that contain the Asp-Pro dipeptide sequence, peptides that contain amino acids that are modified by phosphorylation inside the cell, or peptides that contain an internal methionine are
20 particularly preferred for use in reporter signal calibration. Alternatively, tryptic peptides terminating in arginine may be modified by reaction with acetylacetone (pentane-2,4-dione) to increase the frequency of fragment ions (Dikler et al., J Mass Spectrom 32:1337-49 (1997)). Selection of the subsets of protein fragments can be performed using bioinformatics in order to maximize the information content of the
25 protein signatures.

For this form of reporter signal protein calibration, the protein digest can be mixed with a specially designed set of reporter signal calibrators, and then is analyzed using tandem mass spectrometry. In the case of a tandem in space instrument (for example, Q-ToF™ from Micromass), using first quadrupole settings for single-ion
30 filtering (defined by the molecular mass of each unique fragment, which can be obtained from sequence data), followed by a collision stage for ion fragmentation, and finally TOF, spectrometry of the peptide fragments (generated by cleavage at fragile

bonds, such as Asp-Pro, bonds involving a phosphorylated amino acid, or bonds adjacent to an oxidized amino-acid such as methionine sulfoxide, Smith et al., Free Radic Res. 26:103-11 (1997)) that arise from the original single-ion. In the second stage, signal to noise of the TOF measurement is much larger than in a conventional MS experiment. In general, one reporter signal calibrator can be used for each protein fragment in the sample that will be used to make up the protein signature (such protein fragments are referred to as signature protein fragments), and each is designed to fragment in an easily detectable pattern of masses, distinct from the fragment masses of the unfragmented signature protein fragments. The quadrupole filtering settings are then varied in sequence over a range of values (fifty, for example), corresponding to the masses of each of the protein fragments previously chosen to comprise the protein signature (that is, the signature protein fragments). At each filtered mass setting, there will be two types of signals detectable by TOF after fragmentation, one set derived from the tryptic peptide (that is, the original protein fragment), and another set corresponding to the reporter signal calibrator. The reporter signal calibrator permits one to calculate relative abundance for each of the signature protein fragments. These relative abundance ratios, determined for a given sample, constitute the protein signature. The detection limit of the tandem mass spectrometer in MS/MS mode, is remarkably good, perhaps of the order of 500 molecules of peptide. This level of detection is approximately 1,000 times better than that for MALDI-TOF mass spectrometry, and should permit the generation of protein signatures from single cells.

As can be seen, for this form of reporter signal calibration, the availability of the sequence of the entire human genome, as well as the genomes of many other organisms, can facilitate the identification of protein fragments that are unique in the context of all known proteins. That is, the sequence information can be used to identify peptides that will be generated in a protein signature and guide selection of reporter signal calibrators.

D. Reporter Signal Fusions

In another form of the disclosed method and compositions, referred to as reporter signal fusions (RSF), reporter signal peptides are joined with a protein or peptide of interest in a single amino acid segment. Such fusions of proteins and peptides of interest with reporter signal peptides can be expressed as a fusion protein or

peptide from a nucleic acid molecule encoding the amino acid segment that constitutes the fusion. The fusion protein or peptide is referred to herein as a reporter signal fusion. The reporter signal peptides, a form of reporter signal, allow for sensitive monitoring and detection of the proteins and peptides to which they are fused. In particular, the reporter signal fusions allow sensitive and multiplex detection of expression of particular proteins and peptides of interest, and/or of the genes, vectors, and expression constructs encoding the proteins and peptides of interest. The disclosed reporter signal fusions can also be used for any purpose including as a source of reporter signals for other forms of the disclosed method and compositions.

The disclosed reporter signal fusions also are useful for creating cells, cell lines, and organisms that have particular protein(s), gene(s), vector(s), and/or expression sequence(s) labeled (that is, associated with or involved in) reporter signal fusions. For example, a set of nucleic acid constructs, each encoding a reporter signal fusion with a different reporter signal peptide, can be used to uniquely label a set of cells, cell lines, and/or organisms. Processing of a sample from any of the labeled sources can result in a unique altered form of the reporter signal peptide (or the amino acid segment or an amino acid subsegment) for each of the possible sources that can be distinguished from the other altered forms. Detection of a particular altered form identifies the source from which it came. As a more specific example, a nucleic acid construct encoding a reporter signal fusion can be introduced into a genetically modified plant line (for example, a Roundup resistant corn line) and the plant line can then be identified by detecting the reporter signal fusion. Preferred reporter signal peptides for use in reporter signal fusions used in or associated with different genes, proteins, vectors, constructs, cells, cell lines, or organisms would be those using differentially distributed mass. In particular, the use of alternative amino acid sequences using the same amino acid composition is preferred.

The disclosed reporter signal fusions also can be used to "label" particular pathways, regulatory cascades, and other suites of genes, proteins, vectors, and/or expressions sequences. Such labeling can be within the same cell, cell line, or organism, or across a set of cells, cell lines, or organisms. For example, nucleic acid segments encoding reporter signal fusions can be associated with endogenous expression sequences of interest, endogenous genes of interest, exogenous expression

sequences of interest, exogenous genes of interest, or a combination. The exogenous constructs then are introduced into the cells or organisms of interest. The association with endogenous expression sequences or genes can be accomplished, for example, by introducing a nucleic acid molecule (encoding the reporter signal fusion) for insertion at the site of the expression sequences or gene of interest, or by creating a vector or other nucleic acid construct (containing both the endogenous expression sequences or gene and a nucleic acid segment encoding the reporter signal fusion) in vitro and introducing the construct into the cells or organisms of interest. Many other uses and modes of use are possible, a number of which are described in the illustrations below.

The disclosed reporter signal fusions can be used, for example, in any context and for any purpose that green fluorescent protein and green fluorescent protein fusions are used. However, the disclosed reporter signal proteins have more uses and are more useful than green fluorescent protein at least because of the ability to multiplex more highly the disclosed reporter signal fusions.

Nucleic acid sequences encoding reporter signal peptides can be engineered into particular exons of a gene. This would be the normal situation when the gene encoding the protein to be fused contains introns, although sequence encoding a reporter signal peptide can be split between different exons to be spliced together. Placement of nucleic acid sequences encoding reporter signal peptides into particular exons is useful for monitoring and analyzing alternative splicing of RNA. The appearance of a reporter signal peptide in the final protein indicates that the exon encoding the reporter signal peptide was spliced into the mRNA.

The reporter signal peptides can be used for sensitive detection of one or multiple proteins (that is, the proteins to which the reporter signal peptides are fused).

In the method, proteins fused with reporter signal peptides are analyzed using the reporter signal peptides to distinguish the reporter signal fusions. Detection of the reporter signal peptides indicates the presence of the corresponding protein(s). The detected protein(s) can then be analyzed using known techniques. The reporter signal fusions provide a unique protein/label composition that can specifically identify the protein(s). This is accomplished through the use of the specialized reporter signal peptides as the labels.

Although reference is made above and elsewhere herein to detection of, and fusion with, a "protein" or "proteins," the disclosed method and compositions encompass proteins, peptides, and fragments of proteins or peptides. Thus, reference to a protein herein is intended to refer to proteins, peptides, and fragments of proteins or peptides unless the context clearly indicates otherwise. As used herein "reporter signal fusion" refers to a protein, peptide, or fragment of a protein or peptide to which a reporter signal peptide is fused (that is, joined by peptide bond(s) in the same polypeptide chain) unless the context clearly indicates otherwise. The reporter signal fusion(s) can be fragmented, such as by protease digestion, prior to analysis. An expression sample to be analyzed can also be subjected to fractionation or separation to reduce the complexity of the samples. Fragmentation and fractionation can also be used together in the same assay. Such fragmentation and fractionation can simplify and extend the analysis of the expression. The reporter signal peptide(s) can be fused to a protein in any arrangement, such as at the N-terminal end of the protein, at the C-terminal end of the protein, in or at domain junctions, or at any other appropriate location in the protein. In some forms of the method, it is desirable that the protein remain functional. In such cases, terminal fusions or inter-domain fusions are preferable. Those of skill in the art of protein fusions generally know how to design fusions where the protein of interest is expected to remain functional. In other embodiments, it is not necessary that the protein remain functional in which case the reporter signal peptide and protein can have any desired structural organization.

The reporter signal fusions can be produced by expression from nucleic acid molecules encoding the fusions. Thus, the disclosed fusions generally can be designed by designing nucleic acid segments that encode amino acid segments where the amino acid segments comprise a reporter signal peptide and a protein or peptide of interest. A given nucleic acid molecule can comprise one or more nucleic acid segments. A given nucleic acid segment can encode one or more amino acid segments. A given amino acid segment can include one or more reporter signal peptides and one or more proteins or peptides of interest. The disclosed amino acid segments consist of a single, contiguous polypeptide chain. Thus, although multiple amino acid segments can be part of the same contiguous polypeptide chain, all of the components (that is, the

reporter signal peptide(s) and protein(s) and peptide(s) of interest) of a given amino acid segment are part of the same contiguous polypeptide chain.

Reporter signal peptides can be fragmented, decomposed, reacted, derivatized, or otherwise modified, preferably in a characteristic way. This allows a protein to which the reporter signal peptide is fused to be identified by detection of one or more of the products of the reporter signal fusion following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal peptide. The protein can also be identified by the correlated detection of the reporter signal fusion and one or more of the products of the reporter signal fusion following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal peptide. The alteration of the reporter signal peptide will alter the reporter signal fusion in a characteristic and detectable way. Together, the detection of a characteristic reporter signal fusion and a characteristic product of (that is, altered form of) the reporter signal fusion can uniquely identify the protein (although the altered form alone can be detected, if desired). In this way, using the disclosed method and materials, expression of one or more proteins can be detected, either alone or together (for example, in a multiplex assay). Further, expression of one or more proteins in one or more samples can be detected in a multiplex manner. Preferably, for mass spectrometry reporter signals, the reporter signal peptides are fragmented to yield fragments of similar charge but different mass.

Preferably, the reporter signal peptides are fragmented to yield fragments of similar charge but different mass. This allows each reporter signal fusion (and/or each reporter signal peptide) in a set to be distinguished by the different mass-to-charge ratios of the fragments of (that is, altered forms of) the reporter signal peptides. This is possible since the fragments of the different reporter signal peptides (or the fragments of the reporter signal fusions) can be designed to have different mass-to-charge ratios. In the disclosed method, this allows each reporter signal fusion to be distinguished by the mass-to-charge ratios of the reporter signal fusions after fragmentation of the reporter signal peptide.

Alteration of reporter signal peptides in reporter signal fusions can produce a variety of altered compositions. Any or all of these altered forms can be detected. For example, the altered form of the reporter signal peptide can be detected, the altered

form of the amino acid segment (which contains the reporter signal peptide) can be detected, the altered form of a subsegment of the amino acid segment can be detected, or a combination of these can be detected. Where the reporter signal peptide is altered by fragmentation, the result generally will be a fragment of the reporter signal peptide and an altered form of the amino acid segment containing the protein or peptide of interest and a portion of the reporter signal peptide (that is, the portion not in the reporter signal peptide fragment).

The protein or peptide of interest also can be fragmented. The result would be a subsegment of the amino acid segment. The amino acid subsegment would contain the reporter signal peptide and a portion of the protein or peptide of interest. When the reporter signal peptide in an amino acid subsegment is altered (which can occur before, during, or after fragmentation of the amino acid segment), the result is an altered form of the amino acid subsegment (and an altered form of the reporter signal peptide). This altered form of amino acid subsegment can be detected. Where the reporter signal peptide is altered by fragmentation, the result generally will be a fragment of the reporter signal peptide and an altered form of (that is, fragment of) the amino acid subsegment. In this case, the altered form of the amino acid subsegment will contain a portion of the protein or peptide of interest and a portion of the reporter signal peptide (that is, the portion not in the reporter signal peptide fragment).

As with reporter signals generally, reporter signal peptides can be used in sets where the reporter signal peptides in a set can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. In the case of reporter signal fusions, amino acid segments and amino acid subsegments can be used in sets where the amino acid segments and amino acid subsegments in a set can have one or more common properties that allow the amino acid segments and amino acid subsegments, respectively, to be separated or distinguished from molecules lacking the common property. In general, the component(s) of the reporter signal fusions having common properties can depend on the component(s) to be detected and/or the mode of the method being used.

Nucleic acid molecules encoding reporter signal fusions can be used in sets where the reporter signal peptides in the reporter signal fusions encoded by a set of

nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Similarly, nucleic acid molecules encoding amino acid segments can be used in sets where the reporter signal peptides in the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Nucleic acid molecules encoding amino acid segments can be used in sets where the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the amino acid segments to be separated or distinguished from molecules lacking the common property.

Nucleic acid segments (which, generally, are part of nucleic acid molecules) encoding reporter signal fusions can be used in sets where the reporter signal peptides in the reporter signal fusions encoded by a set of nucleic acid segments can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Similarly, nucleic acid segments encoding amino acid segments can be used in sets where the reporter signal peptides in the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Nucleic acid segments encoding amino acid segments can be used in sets where the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the amino acid segments to be separated or distinguished from molecules lacking the common property. Other relationships between members of the sets of nucleic acid molecules, nucleic acid segments, amino acid segments, reporter signal peptides, and proteins of interest are contemplated.

Reporter signal fusions can include other components besides a protein of interest and a reporter signal peptide. For example, reporter signal fusions can include epitope tags or flag peptides (see, for example, Brizzard et al. (1994) Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. Biotechniques 16:730-735). Epitope tags and flag peptides can serve as tags by which reporter signal fusions can be manipulated.

The use of epitope tags and flag peptides generally is known and can be adapted for use in the disclosed reporter signal fusions.

5 In preferred embodiments, reporter signal peptides, reporter signal fusions (or amino acid segments), nucleic acid segments encoding reporter signal fusion, and/or nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions are used in sets where the reporter signal peptides, the reporter signal fusions, and/or subsegments of the reporter signal fusions constituting or present in the set have similar properties (such as similar mass-to-charge ratios). The similar properties allow the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions constituting or present in a set have the same mass-to-charge ratio (m/z). That is, the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions in a set are isobaric. This allows the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions to be separated precisely from other molecules based on mass-to-charge ratio. The result of the filtering is a huge increase in the signal to noise ratio (S/N) for the system, allowing more sensitive and accurate detection.

20 Cells, cell lines, organisms, and expression of genes and proteins can be detected using the disclosed reporter signal fusions in a variety of ways. For example, the protein and attached reporter signal peptide can be detected together, one or more peptides of the protein and the attached reporter signal peptide(s) can be detected together, the fragments of the reporter signal peptide can be detected, or a combination. Preferred detection involves detection of the reporter signal fusion both before and after fragmentation of the reporter signal peptide.

25 A preferred form of the disclosed method involves correlated detection of the reporter signal peptides both before and after fragmentation of the reporter signal peptide. This allows genes, proteins, vectors, and expression constructs "labeled" with a reporter signal peptide to be detected and identified via the change in the reporter signal fusion and/or reporter signal peptide. That is, the nature of the reporter signal fusion or reporter signal peptide detected (non-fragmented versus fragmented) identifies the gene, protein, vector, or nucleic acid construct from which it was derived.

Where the reporter signal fusions and reporter signal peptides are detected by mass-to-charge ratio, the change in mass-to-charge ratio between fragmented and non-fragmented samples provides the basis for comparison. Such mass-to-charge ratio detection is preferably accomplished with mass spectrometry.

5 As an example, a fusion between a protein of interest and a reporter signal peptide designed as a mass spectrometry label can be expressed. The reporter signal fusion can be subjected to tryptic digest followed by mass spectrometry of the resulting materials. A peak corresponding to the tryptic fragment containing the reporter signal peptide will be detected. Fragmentation of the reporter signal peptide in the mass
10 spectrometer (preferably in a collision cell) would result in a shift in the peak corresponding to the loss of a portion of the attached reporter signal peptide, the appearance of a peak corresponding to the lost fragment, or a combination of both events. Significantly, the shift observed will depend on which reporter signal peptide is fused to the protein since different reporter signal peptides will, by design, produce
15 fragments with different mass-to-charge ratios. The combination event of detection of the parent mass-to-charge (with no collision gas) and the mass-to-charge corresponding to the loss of the fragment from the reporter signal peptide (with collision gas) indicates a reporter signal fusion (thus indicating expression of the reporter signal fusion and of the gene, vector, or construct encoding it).

20 A powerful form of the disclosed method is use of reporter signal fusions to assay multiple samples (for example, time series assays or other comparative analyses). Knowledge of the temporal response of a biological system following perturbation is a very powerful process in the pursuit of understanding the system. To follow the temporal response a sample of the system is obtained (for example, cells from a cell
25 culture, mice initially synchronized and sacrificed) at determined times following the perturbation. Knowledge of spatial protein profiles (for example, relative position within a tissue section) is a very powerful process in the pursuit of understanding the biological system.

 The reporter signal fusions are preferably detected using mass spectrometry
30 which allows sensitive distinctions between molecules based on their mass-to-charge ratios. A set of isobaric reporter signal peptides or reporter signal fusions can be used for multiplex labeling and/or detection of the expression of many genes, proteins,

vectors, expression constructs, cells, cell lines, and organisms since the reporter signal peptide fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection. Where the same gene, protein, vector, expression construct, cell, cell line, or organism (or the same type of gene, protein, vector, expression construct, cell, cell line, or organism) is labeled with a set of reporter signal fusions that are isobaric or that include isobaric reporter signal peptides (by, for example, "labeling" the same gene, protein, vector, expression construct, cell, cell line, or organism in different samples), the set of reporter signal fusions or reporter signal peptides that results will also be isobaric. Fragmentation of the reporter signal peptides will split the set of reporter signal peptides into individually detectable reporter signal fusion fragments and reporter signal peptide fragments of characteristically different mass.

A preferred form of the disclosed method involves filtering of isobaric reporter signal fusions or reporter signal peptides from other molecules based on mass-to-charge ratio, fragmentation of the reporter signal peptides to produce fragments having different masses, and detection of the different fragments based on their mass-to-charge ratios. The method is best carried out using a tandem mass spectrometer as described elsewhere herein.

Nucleic acid sequences and segments encoding reporter signal fusions can be expressed in any suitable manner. For example, the disclosed nucleic acid sequences and nucleic acid segments can be expressed *in vitro*, in cells, and/or in cells in organism. Many techniques and systems for expression of nucleic acid sequences and proteins are known and can be used with the disclosed reporter signal fusions. For example, many expression sequences, vector systems, transformation and transfection techniques, and transgenic organism production methods are known and can be used with the disclosed reporter signal peptide method and compositions.

For example, kits for the *in vitro* transcription/translation of DNA constructs containing promoters and nucleic acid sequence to be transcribed and translated are known (for example, PROTEINscript-PRO™ from Ambion, Inc. Austin TX; Wilkinson (1999) "Cell-Free And Happy: In Vitro Translation And Transcription/Translation Systems", The Scientist 13[13]:15, Jun. 21, 1999). Such constructs can be used in the genomic DNA of an organism, in a plasmid or other

- vector that may be transfected into an organism, or in *in vitro* systems. For example, constructs containing a promoter sequence and a nucleic acid sequence that, following transcription and translation, results in production of green fluorescence protein or luciferase as a reporter/marker in *in vivo* systems are known (for example, Sawin and Nurse, "Identification of fission yeast nuclear markers using random polypeptide fusions with green fluorescent protein." *Proc Natl Acad Sci U S A* 93(26): 15146-51 (1996); Chatterjee et al., "In vivo analysis of nuclear protein traffic in mammalian cells." *Exp Cell Res* 236(1):346-50 (1997); Patterson et al., "Quantitative imaging of TATA-binding protein in living yeast cells." *Yeast* 14(9):813-25 (1998);
- 10 Dhandayuthapani et al., "Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages." *Mol Microbiol* 17(5):901-12 (1995); Kremer et al., "Green fluorescent protein as a new expression marker in mycobacteria." *Mol Microbiol* 17(5):913-22 (1995); Reiländer et al., "Functional expression of the *Aequorea victoria* green fluorescent protein in insect cells
- 15 using the baculovirus expression system." *Biochem Biophys Res Commun* 219(1): 14-20 (1996); Mankertz et al., "Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma" *J Cell Sci*, 113:2085-90 (2000); White et al., "Real-time analysis of the transcriptional regulation of HIV and hCMV promoters in single mammalian cells" *J Cell Sci*, 108:441-55 (1995)). Green
- 20 fluorescence protein, or variants, have been shown to be stably incorporated and not interfere with the organism — generally GFP is larger relative to the disclosed reporter signal peptides (GFP from *Aequorea Victoria* is 238 amino acids in size; NCBI GI:606384), and thus the generally smaller reporter signal peptides are less likely to disrupt an expression system to which they are added.
- 25 Techniques are known for modifying promoter regions such that the endogenous promoter is replaced with a promoter-reporter construct, for example, where the reporter is green fluorescent protein (Patterson et al., "Quantitative imaging of TATA-binding protein in living yeast cells." *Yeast* 14(9): 813-25 (1998)) or luciferase. Transcription factor concentrations are followed by monitoring the GFP or
- 30 luciferase. These techniques can be used with the disclosed reporter signal fusions and reporter signal fusion constructs. Techniques are also known for targeted knock-in of nucleic acid sequences into a gene of interest, typically under control of the endogenous

promoter. Such techniques, which can be used with the disclosed method and compositions, have been used to introduce reporter/markers of the transcription and translation of the gene where the nucleic acid was inserted. The same techniques can be used to place the disclosed reporter signal fusions under control of endogenous expression sequences. Alternately, non-targeted knock-ins (techniques for which are also known; Hobbs et al. "Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins" *Biochem Biophys Res Commun*, 252:368-72 (1998); Kershner et al., "Immunoaffinity purification and functional characterization of human transcription factor IIIH and RNA polymerase II from clonal cell lines that conditionally express epitope-tagged subunits of the multiprotein complexes" *J Biol Chem*, 273:34444-53 (1998); Wu and Chiang, "Establishment of stable cell lines expressing potentially toxic proteins by tetracycline-regulated and epitope-tagging methods" *Biotechniques* 21:718-22, 724-5 (1996)) can be used to follow the level or activity of transcription factors—reporter signal peptide fusions associated with the inserted nucleic acid code can directly indicate the transcription/translation activity.

The disclosed reporter signal fusions also can be used in the detection and analysis of protein interactions with other proteins and molecules. For example, interaction traps for protein-protein interactions include the well known yeast two-hybrid (Fields and Song, "A novel genetic system to detect protein-protein interactions" *Nature* 340:245-6 (1989); Uetz et al., "A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*" *Nature* 403:623-7 (2000)) and related systems (Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., 2001; Van Crielinge and Beyaert, "Yeast two-hybrid: state of the art" *Biological Procedures Online*, 2(1), 1999). Incorporation of nucleic acid sequence encoding a peptide reporter signal can be introduced into these systems, for example at a terminus of the ordinarily used LacZ selection region (LacZ selection is described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, second edition, 1989, Cold Spring Harbor Laboratory Press, New York). A set of such incorporated sequences (for example, in a set of such plasmids, where each plasmid has a reporter signal coding sequence and the LacZ functionality), allows the unambiguous detection

of many interactions simultaneously rather (as many different interactions as reporter signals used).

In another mode of reporter signal fusions, a nucleic acid sequence encoding a reporter signal could be added to sequence encoding the constant (C) region of T cell and B cell receptors. The reporter signal would appear in T or B cell receptors when that C region is spliced to a J region following transcription.

In another mode of reporter signal fusions, referred to as reporter signal presentation, the presentation of specific antigenic peptides by major histocompatibility (MHC) and non-major histocompatibility molecules can be detected and analyzed. It is well known that protein antigens are processed by antigen presenting cells and that small peptides, typically 8-12 amino acids are presented by Class I and Class II MHC molecules for recognition by T cells. The study of specific T cell/peptide-MHC complexes is technically challenging due various labeling requirements (either radioactive or fluorescence) and the common reliance on antibody reagents that recognize specific receptors and/or peptide-MHC complexes.

There is a need to be able to further expand our knowledge of antigen processing and antigen presentation. Reporter signals that have been engineered into specific protein antigens could provide novel insight into this process and enable new experimental approaches. For instance, consider two viral or bacterial proteins, protein A and protein B, that differ by only a few amino acids. It would be useful to know if they are processed and presented to immune cells (for example, T cells) with the same efficiency. By engineering reporter signals into protein A and engineered protein B to antigen presenting cells, one could test for the presence of the different reporter signals presented on and thus determine if the proteins are efficiently processed and presented. The presence of reporter signal A (present in protein A) but not reporter signal B (present in protein B), indicates that protein A is processed and that protein B is not. The lack of antigen processing of protein B may then be an explanation of why a virus or bacteria escapes immune surveillance by the immune system. Antigenic peptides are characterized by conserved anchor residues near both the amino and carboxy ends, with more heterogeneity tolerated in the middle. This middle heterogeneity is thus a preferred site for addition of a reporter signal peptide.

E. Rearranging Reporter Signals

Another embodiment of the disclosed method and compositions, referred to as rearranging reporter signals, enables one to detect the occurrence of specific gene rearrangement events, their protein products, and specific cell populations bearing those
5 receptors. Rearranging reporter signals will also allow one to follow the progression or development of certain receptors and cells or populations of cells by monitoring the presence and/or absence of a reporter signal. Design considerations for rearranged reporter signals are analogous to those required for reporter signal fusions as described elsewhere herein.

10 Most embodiments of the disclosed method involve intact reporter signals that are associated with analytes in various ways. Rearranging reporter signals make use of processes, such as biological processes, to form reporter signals by specific rearrangement of the reporter signal pieces or rearrangement of nucleic acid segments encoding only portions of reporter signals. One form of rearranging reporter signals
15 utilizes endogenous biological systems, such as the variable-diversity-joining (V-D-J) gene rearrangement machinery present in the mammalian immune system. In this system, short stretches of germline DNA (the V, D & J gene fragments) that are not contiguous, are brought together (recombined) prior to serving as a template for transcription. Gene rearrangement occurs in white blood cells such as T and B
20 lymphocytes and is a key mechanism for generating diversity of T cell and B cell antigen receptors. Theoretically, billions of different receptors can be generated. This level of complexity makes it difficult to detect the presence of rare rearrangement events, or receptors. PCR based assays and flow cytometry approaches are now used to study receptor diversity. However, PCR approaches are laborious and do not provide
25 any information on the status of expressed protein. Flow cytometry approaches have limited multiplexing capabilities due to emission spectra overlap of the fluorescent probes used.

If one desired to test for 50-100 T cell or B cell receptors, one would need to make use of a similar number of antibodies to those receptors, something that in
30 practice is not done. Therefore, there is a real need for methods that would allow highly sensitive and specific detection of specific receptors in a highly complex pool of receptors. The ability to highly multiplex this approach would enable currently

unattainable experimental approaches. The disclosed reporter signal technology allows large scale multiplexing of signals for detection.

As an example of rearranging reporter signals, transgenic mice can be generated in which nucleic acid sequences encoding reporter signals have been engineered into the mouse germline. Methods for doing this are well known in the art and include using standard molecular biology methods to engineer rearranging reporter signal into, for example, yeast or bacterial artificial chromosomes (YACs or BACs) and then using these constructs to generate transgenic mice.

As an example of the use of immunoglobulin rearrangement for rearranging reporter signals, part of a reporter signal could be encoded on the D region and another part of the reporter signal could be encoded on the J region. Upon a rearrangement event that joined the D and J regions encoding these "partial" reporter signals, a coding sequence for a "complete" reporter signal would be generated. Following transcription and translation, the reporter signal would be encoded within the protein product. The reporter signal could then be detected as described elsewhere herein. In the absence of a rearrangement event that joins the engineered D and J region, no reporter signal would be detected. By including sequences encoding parts of a variety of reporter signals with different D and J regions, a variety of different reporter signals can be generated by rearrangement, a different, and diagnostic, reporter signal for each of the different possible rearrangements. This system also could be extended to include, for example, reporter signals split among three or more gene regions (for example, V-D-J, V-D-D-J, etc) with the result that multiple rearrangement events would produce the reporter signal. In this mode, the combinations of rearrangements of the reporter signal parts can give rise to an large number of different reporter signals, each characterized by the specific reporter signal parts rearranged to form the reporter signal.

Transgenic mice carrying rearranging reporter signals would enable one to address questions that would otherwise be very difficult or impossible to address. For instance, one could dissect what specific T and B cell receptors (out of the thousands or millions possible) respond to specific stimuli or what cell types are present at certain stages of development.

F: Mass Spectrometers

The disclosed methods can make use of mass spectrometers for analysis of reporter signals, altered forms of reporters signals, and various analytes and analyte fragments. Mass spectrometers are generally available and such instruments and their operations are known to those of skill in the art. Fractionation systems integrated with mass spectrometers are commercially available, exemplary systems include liquid chromatography (LC) and capillary electrophoresis (CE).

The principle components of a mass spectrometer include: (a) one or more sources, (b) one or more analyzers and/or cells, and (c) one or more detectors. Types of sources include Electrospray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI). Types of analyzers and cells include quadrupole mass filter, hexapole collision cell, ion cyclotron trap, and Time-of-Flight (TOF). Types of detectors include Multichannel Plates (MCP) and ion multipliers. A preferred mass spectrometer for use with the disclosed method is described by Krutchinsky et al., Rapid Automatic Identification of Proteins Utilizing a Novel MALDI-Ion Trap Mass Spectrometer, Abstract of the 49th ASMS Conference on Mass Spectrometry and Allied Topics (May 27-31, 2001), The Rockefeller University, New York, New York.

Mass spectrometers with more than one analyzer/cell are known as tandem mass spectrometers. There are two types of tandem mass spectrometers, as well as hybrids and combinations of these types: "tandem in space" spectrometers and "tandem in time" spectrometers. Tandem mass spectrometers where the ions traverse more than one analyzer/cell are known as tandem in space mass spectrometers. Tandem in space spectrometers utilize spatially ordered elements and act upon the ions in turn as the ions pass through each element. Tandem mass spectrometers where the ions remain primarily in one analyzer/cell are known as tandem in time mass spectrometers. Tandem in time spectrometers utilize temporally ordered manipulations on the ions as the ions are contained in a space. Hybrid systems and combinations of these types are known. The ability to select a particular mass-to-charge ratio of interest in a mass analyzer is typically characterized by the resolution (reported as the centroid mass-to-charge divided by the full width at half maximum of the selected ions of interest). Thus resolution is an indicator of the narrowness of the ion mass-to-charge distribution passed through the analyzer to the detector. Reference to such resolution is generally

noted herein by referring to the ability of a mass spectrometer to pass only a narrow range of mass-to-charge ratios.

A preferred form of mass spectrometer for use in the disclosed methods is a tandem mass spectrometer, such as a tandem in space tandem mass spectrometer. As
5 an example of the use of a tandem in space class of instrument, the isobaric reporter signals can be first passed through a filtering quadrupole, the reporter signals are fragmented (preferably in a collision cell), and the fragments are distinguished and detected in a time-of-flight (TOF) stage. In such an instrument the sample is ionized in the source (for example, in a MALDI ion source) to produce charged ions. It is
10 preferred that the ionization conditions are such that primarily a singly charged parent ion is produced. A first quadrupole, Q0, is operated in radio frequency (RF) mode only and acts as an ion guide for all charged particles. The second quadrupole, Q1, is operated in RF + DC mode to pass only a narrow range of mass-to-charge ratios (that includes the mass-to-charge ratio of the reporter signals). This quadrupole selects the
15 mass-to-charge ratio of interest. Quadrupole Q2, surrounded by a collision cell, is operated in RF only mode and acts as ion guide. The collision cell surrounding Q2 can be filled to appropriate pressure with a gas to fracture the input ions by collisionally induced dissociation when fragmentation of the reporter signals is desired. The collision gas preferably is chemically inert, but reactive gases can also be used.
20 Preferred molecular systems utilize reporter signals that contain scissile bonds, labile bonds, or combinations, such that these bonds will be preferentially fractured in the Q2 collision cell.

Tandem instruments capable of MS^N can be used with the disclosed method. As an example consider, a method where one selects a set of molecules using a first
25 stage filter (MS), photocleaves these molecules to yield a set of reporter signals, selects these reporter signals using a second stage (MS/MS), alters these reporter signals by collisional fragmentation, detects by time of flight (MS3). Many other combinations are possible and the disclosed method can be adapted for use with such systems. For example, extension to more stages, or analysis of reporter signal fragments is within the
30 skill of those in the art.

Materials

A. Reporter Signals

Reporter signals are molecules that can be preferentially fragmented, decomposed, reacted, derivatized, or otherwise modified or altered for detection.

- 5 Detection of the modified reporter signals is preferably accomplished with mass spectrometry. The disclosed reporter signals are preferably used in sets where members of a set have the same mass-to-charge ratio (m/z). This facilitates sensitive filtering or separation of reporter signals from other molecules based on mass-to-charge ratio. Reporter signals can have any structure that allows modification of the reporter
10 signal and identification of the different modified reporter signals. Reporter signals preferably are composed such that at least one preferential bond rupture can be induced in the molecule. A set of reporter signals having nominally the same molecular mass and arbitrarily chosen internal fragmentation points may be constructed such that upon fragmentation each member of the set will yield unique correlated daughter fragments.
15 For convenience, reporter signals that are fragmented, decomposed, reacted, derivatized, or otherwise modified for detection are referred to as fragmented reporter signals.

- Preferred reporter signals are made up of chains of subunits such as peptides, oligonucleotides, peptide nucleic acids, oligomers, carbohydrates, polymers, and other
20 natural and synthetic polymers and any combination of these. Most preferred chains are peptides, and are referred to herein as reporter signal peptides. Chains of subunits and subunits have a relationship similar to that of a polymers and mers. The mers are connected together to form a polymer. Likewise, subunits are connected together to form chains of subunits. Preferred reporter signals are made up of chains of similar or
25 related subunits. These are termed homochains or homopolymers. For example, nucleic acids are made up of phosphonucleosides and peptides are made up of amino acids.

- Reporter signals can also be made up of heterochains or heteropolymers. A heterochain is a chain or a polymer where the subunits making up the chain are
30 different types or the mers making up the polymer are different types. For example, a heterochain could be guanosine-alanine, which is made up of one nucleoside subunit and one amino acid subunit. It is understood that any combination of types of subunits

can be used within the disclosed compositions, sets, and methods. Any molecule having the required properties can be used as a reporter signal. Preferred reporter signals can be fragmented in tandem mass spectrometry.

Reporter signals preferably are used in sets where all the reporter signals in the set have similar physical properties. The similar (or common) properties allow the reporter signals to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signals in a set have the same mass-to-charge ratio (m/z). That is, the reporter signals in a set are isobaric. This allows the reporter signals (and/or the proteins to which they are attached) to be separated precisely from other molecules based on mass-to-charge ratio. The result of the filtering is a huge increase in the signal to noise ratio (S/N) for the system, allowing more sensitive and accurate detection. Sets of reporter signals can have any number of reporter signals. For example, sets of reporter signals can have one, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, one hundred or more, two hundred or more, three hundred or more, four hundred or more, or five hundred or more different reporter signals. Although specific numbers of reporter signals and specific endpoints for ranges of the number of reporter signals are recited, each and every specific number of reporter signals and each and every specific endpoint of ranges of numbers of reporter signals are specifically contemplated, although not explicitly listed, and each and every specific number of reporter signals and each and every specific endpoint of ranges of numbers of reporter signals are hereby specifically described.

The sets of reporter signals can be made up of reporter signals that are made up of chains or polymers. The set of reporter signals can be homosets which means that the set is made up of one type of reporter signal or that the reporter signal is made up of homochains or homopolymers. The set of reporter signals can also be a heteroset which means that the set is made up of different reporter signals or of reporter signals that are made up of different types of chains or polymers. A special type of heteroset is one in which the set is made up of different homochains or homopolymers, for example one peptide chain and one nucleic acid chain. Another special type of heteroset is one where the chains themselves are heterochains or heteropolymers. Still another type of

heteroset is one which is made up of both heterochains/heteropolymers and homochains/homopolymers.

A variety of different properties can be used as the common physical property used to separate reporter signals from other molecules lacking the common property.

- 5 For example, other physical properties useful as common properties include mass, charge, isoelectric point, hydrophobicity, chromatography characteristics, and density. It is preferred that the physical property shared by reporter signals in a set (and used to distinguish or separate the reporter signals from other molecules) is an overall property of the reporter signal (for example, overall mass, overall charge, isoelectric point, 10 overall hydrophobicity, etc.) rather than the mere presence of a feature or moiety (for example, an affinity tag, such as biotin). Such properties are referred to herein as "overall" properties (and thus, reporter signals in a set would be referred to as sharing a "common overall property"). It should be understood that reporter signals can have features and moieties, such as affinity tags, and that such features and moieties can 15 contribute to the common overall property (by contributing mass, for example). However, such limited and isolated features and moieties would not serve as the sole basis of the common overall property.

- A preferred common overall property is the property of subunit isomers. This property occurs when a set of at least two reporter signals (which typically are made up 20 of subunit chains which are in turn made up of subunits, for example, like the relationship between a polymer and the units that make up a polymer) is made up of subunit isomers, and the set could then be called subunit isomeric or isomeric for subunits. Subunits are discussed elsewhere herein, but reporter signals can be made up of any type of chain, such as peptides or nucleic acids or polymer (general) which are 25 in turn made up of subunits for example amino acids and phosphonucleosides, and mers (general) respectively. Within each type of subunit there are typically multiple members that are all the same type of subunit, but differ. For example, within the subunit type "amino acids," there are many members, for example, ala, tyr, and ser, or any other combination of amino acids.

- 30 When a set of reporter signals is subunit isomeric or is made up of subunit isomers this means that each individual of the set is a subunit isomer of every other individual subunit in the set. Isomer or isomeric means that the makeup of the subunits

forming the subunit chain (i.e., distribution or array) is the same but the overall connectivity of the subunits, forming the chain, is different. Thus, for example, a first reporter signal could be the chain, ala-ser-lys-gln, a second reporter signal could be the chain ala-lys-ser-gln, and a third reporter signal could be the chain ala-ser-lys-pro. If a set of reporter signals was made that contained the first reporter signal and the second reporter signal, the set would be subunit isomeric because the first reporter signal and the second reporter signal have the same makeup, i.e. each has one ala, one ser, one lys, and one gln, but each chain has a different connectivity. If, however, the set of reporter signals were made which contained the first, second, and third reporter signals the set would not be isomeric because the make up of each chain would not be the same because the first and second chains do not have a pro and the third chain does not have a gln.

Another illustration is the following: a first reporter signal could be the chain, ala-guanosine-lys-adenosine, a second reporter signal could be the chain ala-adenosine-lys-guanosine, and a third reporter signal could be the chain ala-ser-lys-pro. If a set of reporter signals was made that contained the first reporter signal and the second reporter signal, the set would be subunit isomeric because the first reporter signal and the second reporter signal have the same makeup, i.e. each has one ala, one guanosine, one lys, and one adenosine, but each chain has a different connectivity. If, however, the set of reporter signals were made which contained the first, second, and third reporter signals the set would not be isomeric because the makeup of each chain would not be the same because the first and second chains do not have a pro or a ser and the third chain does not have a guanosine or adenosine. This illustration shows that the sets can be made up of, or include, heterochains and still be considered subunit isomers.

It is preferred that the common property of reporter signals is not an affinity tag. Nevertheless, even in such a case, reporter signals that otherwise have a common property may also include an affinity tag—and in fact may all share the same affinity tag—so long as another common property is present that can be (and, in some embodiments of the disclosed method, is) used to separate reporter signals sharing the common property from other molecules lacking the common property. With this in mind, it is preferred that, if chromatography or other separation techniques are used to separate reporter signals based on the common property, the affinity be based on an

overall physical property of the reporter signals and not on the presence of, for example, a feature or moiety such as an affinity tag. As used herein, a common property is a property shared by a set of components (such as reporter signals). That is, the components have the property "in common." It should be understood that reporter signals in a set may have numerous properties in common. However, as used herein, the common properties of reporter signals referred to are only those used in the disclosed method to distinguish and/or separate the reporter signals sharing the common property from molecules that lack the common property.

Reporter signals in a set can be fragmented, decomposed, reacted, derivatized, or otherwise modified or altered to distinguish the different reporter signals in the set. Preferably, the reporter signals are fragmented to yield fragments of similar charge but different mass. The reporter signals can also be fragmented to yield fragments of different charge and mass. Such changes allow each reporter signal in a set to be distinguished by the different mass-to-charge ratios of the fragments of the reporter signals. This is possible since, although the unfragmented reporter signals in a set are isobaric, the fragments of the different reporter signals are not. Thus, a key feature of the disclosed reporter signals is that the reporter signals have a similarity of properties while the modified reporter signals are distinguishable.

Differential distribution of mass in the fragments of the reporter signals can be accomplished in a number of ways. For example, reporter signals of the same nominal structure (for example, peptides having the same amino acid sequence), can be made with different distributions of heavy isotopes, such as deuterium (^2H), tritium (^3H) ^{17}O , ^{18}O , ^{13}C , or ^{14}C ; stable isotopes are preferred. All reporter signals in the set would have the same number of a given heavy isotope, but the distribution of these would differ for different reporter signals. An example of such a set of reporter signals is A*G*SLDPAGSLR, A*GSLDPAG*SLR, and AGSLDPA*G*SLR (SEQ ID NO:2), where the asterisk indicates at least one heavy isotope substituted amino acid. For a singly charged parent ion and, following fragmentation at the scissile DP bond, one predominantly charged daughter, there are three distinguishable primary daughter ions, PAGSLR⁺, PAG*SLR⁺, PA*G*SLR⁺ (amino acids 6-11 of SEQ ID NO:2).

Similarly, reporter signals of the same general structure (for example, peptides having the same amino acid sequence), can be made with different distributions of

modifications or substituent groups, such as methylation, phosphorylation, sulphation, and use of seleno-methionine for methionine. All reporter signals in the set would have the same number of a given modification, but the distribution of these would differ for different reporter signals. An example of such a set of reporter signals is

- 5 AGS*M*LDPAGSMLR, AGS*MLDPAGSM*LR, and AGS*MLDPAGS*M*LR (SEQ ID NO:3), where S* indicates phosphoserine rather than serine, and, M* indicates seleno-methionine rather than methionine. For a singly charged parent ion and, following fragmentation at the scissile DP bond, one predominantly charged daughter, there are three distinguishable primary daughter ions, PAGSMLR⁺,
 10 PAGSM*LR⁺, PAGS*M*LR⁺ (amino acids 7-13 of SEQ ID NO:3).

- Reporter signals of the same nominal composition (for example, made up of the same amino acids), can be made with different ordering of the subunits or components of the reporter signal. All reporter signals in the set would have the same number of subunits or components, but the distribution of these would be different for different
 15 reporter signals. An example of such a set of reporter signals is AGSLADPGSLR (SEQ ID NO:4), ALSLADPGSGR (SEQ ID NO:5), ALSLGDPASGR (SEQ ID NO:6). For a singly charged parent ion and, following fragmentation at the scissile DP bond, one predominantly charged daughter, there are three distinguishable primary daughter ions, PGSLR⁺ (amino acids 7-11 of SEQ ID NO:4), PGSGR⁺ (amino acids 7-11 of
 20 SEQ ID NO:5), PASGR⁺ (amino acids 7-11 of SEQ ID NO:6).

- Reporter signals having the same nominal composition (for example, made up of the same amino acids), can be made with a labile or scissile bond at a different location in the reporter signal. All reporter signals in the set would have the same number and order of subunits or components. Where the labile or scissile bond is
 25 present between particular subunits or components, the order of subunits or components in the reporter signal can be the same except for the subunits or components creating the labile or scissile bond. Reporter signal peptides used in reporter signal fusions preferably use this form of differential mass distribution. An example of such a set of reporter signals is AGSLADPGSLR (SEQ ID NO:4), AGSDPLAGSLR (SEQ ID
 30 NO:7), ADPGSLAGSLR (SEQ ID NO:8). For a singly charged parent ion and, following fragmentation at the scissile DP bond, one predominantly charged daughter, there are three distinguishable primary daughter ions, PGSLR⁺ (amino acids 7-11 of

SEQ ID NO:4), PLAGSLR⁺ (amino acids 5-11 of SEQ ID NO:7), PGSLAGSLR⁺ (amino acids 3-11 of SEQ ID NO:8).

Each of these modes can be combined with one or more of the other modes to produce differential distribution of mass in the fragments of the reporter signals. For example, different distributions of heavy isotopes can be used in reporter signals where a labile or scissile bond is placed in different locations. Different mass distribution can be accomplished in other ways. For example, reporter signals can have a variety of modifications introduced at different positions. Some examples of useful modifications include acetylation, methylation, phosphorylation, seleno-methionine rather than methionine, sulphation. Similar principles can be used to distribute charge differentially in reporter signals. Differential distribution of mass and charge can be used together in sets of reporter signals.

Reporter signals can also contain combinations of scissile bonds and labile bonds. This allows more combinations of distinguishable signals or to facilitate detection. For example, labile bonds may be used to release the isobaric fragments, and the scissile bonds used to decode the proteins.

Selenium substitution can be used to alter the mass of reporter signals. Selenium can substitute for sulfur in methionine, resulting in the modified amino acid selenomethionine. Selenium is approximately forty seven mass units larger than sulfur. Mass spectrometry may be used to identify peptides or proteins incorporating selenomethionine and methionine at a particular ratio. Small proteins and peptides with known selenium/sulfur ratio are preferably produced by chemical synthesis incorporating selenomethionine and methionine at the desired ratio. Larger proteins or peptides may be produced from an *E. coli* expression system, or any other expression system that inserts selenomethionine and methionine at the desired ratio (Hendrickson *et al.*, *Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure*. *Embo J*, 9(5):1665-72 (1990), Cowie and Cohen, *Biosynthesis by Escherichia coli of active altered proteins containing selenium instead of sulfur*. *Biochimica et Biophysica Acta*, 26:252 – 261 (1957), and Oikawa *et al.*, *Metalloselenonein, the selenium analogue of metallothionein: synthesis and*

characterization of its complex with copper ions. Proc Natl Acad Sci USA, 88(8):3057-9 (1991).

Some forms of reporter signals can include one or more affinity tags. Such affinity tags can allow the detection, separation, sorting, or other manipulation of the labeled proteins, reporter signals, or reporter signal fragments based on the affinity tag. Such affinity tags are separate from and in addition to (not the basis of) the common properties of a set of reporter signals that allows separation of reporter signals from other molecules. Rather, such affinity tags serve the different purpose of allowing manipulation of a sample prior to or as a part of the disclosed method, not the means to separate reporter signals based on the common property. Reporter signals can have none, one, or more than one affinity tag. Where a reporter signal has multiple affinity tags, the tags on a given reporter signal can all be the same or can be a combination of different affinity tags. Affinity tags also can be used to distribute mass and/or charge differentially on reporter tags following the principles described above and elsewhere herein. Affinity tags can be used with reporter signals in a manner similar to the use of affinity labels as described in PCT Application WO 00/11208.

Peptide-DNA conjugates (Olejnik *et al.*, *Nucleic Acids Res.*, 27(23):4626-31 (1999)), synthesis of PNA-DNA constructs, and special nucleotides such as the photocleavable universal nucleotides of WO 00/04036 can be used as reporter signals in the disclosed method. Useful photocleavable linkages are also described by Marriott and Ottl, *Synthesis and applications of heterobifunctional photocleavable cross-linking reagents*, *Methods Enzymol.* 291:155-75 (1998).

Photocleavable bonds and linkages are useful in (and for use with) reporter signals because it allows precise and controlled fragmentation of the reporter signals (for subsequent detection) and precise and controlled release of reporter signals from analytes (or other intermediary molecules) to which they are attached. A variety of photocleavable bonds and linkages are known and can be adapted for use in and with reporter signals. Recently, photocleavable amino acids have become commercially available. For example, an Fmoc protected photocleavable slightly modified phenylalanine (Fmoc-D,L- β Phe(2-NO₂)) is available (Catalog Number 0011-F; Innovachem, Tucson, AZ). The introduction of the nitro group into the phenylalanine ring causes the amino acid to fragment under exposure to UV light (at a wavelength of

approximately 350 nm). The nitrogen laser emits light at approximately 337 nm and can be used for fragmentation. The wavelength used will not cause significant damage to the rest of the peptide.

5 Fmoc synthesis is a common technique for peptide synthesis and Fmoc-derivative photocleavable amino acids can be incorporated into peptides using this technique. Although photocleavable amino acids are usable in and with any reporter signal, they are particularly useful in peptide reporter signals.

10 Use of photocleavable bonds and linkages in and with reporter signals can be illustrated with the following examples. Materials on a blank plastic substrate (for example, a Compact Disk (CD)) may be directly measured from that surface using a MALDI source ion trap. For example, a thin section of tissue sample, flash frozen, could be applied to the CD surface. A reporter molecule (for example, an antibody with a reporter signal attached via a photocleavable linkage) can be applied to the tissue surface. Recognition of specific components within the tissue allows for some of the
15 antibody/reporter signal conjugates to associate (excess conjugate is removed during subsequent wash steps). The reporter signal then can be released from the antibody by applying a UV light and detected directly using the MALDI ion trap instrument. For example, a peptide of sequence CF*XXXXXDPXXXXXR (SEQ ID NO:24)(which contains a reporter signal) can be attached to an antibody using a disulfide bond linkage
20 method. Exposure to the UV source of a MALDI laser will cleave the peptide at the modified phenylalanine, F*, releasing the XXXXXDPXXXXXR reporter signal (amino acids 3-15 of SEQ ID NO:24). The reporter signal subsequently can be fragmented at the DP bond and the charged fragment detected as described elsewhere herein.

25 Another example of the use of photocleavable linkages with reporter signals involves DNA-peptide chimeras used as reporter molecules. Such reporter molecules are useful as probes to detect particular nucleic acid sequences. In a DNA-peptide chimera (or PNA-peptide chimera), the peptide portion can be or include a reporter signal. Placement of a photocleavable phenylalanine, for example, near the DNA peptide junction of the reporter molecule allows for the release of the reporter signal
30 from the reporter molecule by UV light. The released reporter signal can be detected directly or fragmented and detected as described elsewhere herein. Similarly to the case of the antibody-peptide reporter molecule described above, the DNA-peptide

chimera can be associated with a nucleic acid molecule present on the surface of a substrate such as a CD and the reporter signal released using the UV source of a MALDI laser.

5 A photocleavable linkage also can be incorporated into a reporter signal and used for fragmentation of the reporter signal in the disclosed methods. For example, a photocleavable amino acid (such as the photocleavable phenylalanine) can be incorporated at any desired position in a peptide reporter signal. A reporter signal such as XXXXXXXF*XXXXXR containing photocleavable phenylalanine (F*) that is
10 photocleavable. The reporter signal can then be fragmented using the appropriate wavelength of light and the charged fragment detected. When ionizing the reporter signal (from a surface, for example) for detection, a MALDI laser that does not cause significant photocleavage (for example, Er:YAG at 2.94 μm) can be used for ionization and a second laser (for example, Nitrogen at 337 nm) can be used to fragment the reporter signal. In this case XXXXXXXFXXXXXR⁺ would be photocleaved to yield
15 XXXXXR⁺. The second laser may intersect the reporter signal ion packet at any location. Modification to the vacuum system of a mass spectrometer for this purpose is straightforward.

The use of photocleavable linkages in reporter signals is particularly useful when the analyte (or other component) to which the reporter signal is attached could
20 fragment at a scissile bond in a collision cell. For example, in reporter signal fusions, a protein fragment/reporter signal polypeptide could be generated that contained a scissile bond in both the protein fragment portion and the reporter signal portion. An example would be XXXXXXXXXDPXXX(XXXXXXXXDPXXXXXXXXR)XXXX (SEQ ID NO:25), where the sequence in parenthesis indicate the reporter signal portion
25 and the DP dipeptides contain scissile bonds. Fragmenting this polypeptide in a collision cell could result in fragmentation at either or both of the DP bonds, thus complicating the fragment spectrum. Use of a photocleavable linkage (such as a photocleavable amino acid) in the reporter signal portion would allow specific photocleavage of the reporter signal during analysis. For example, an analogous
30 polypeptide XXXXXXXXXDPXXX(XXXXXXF*XXXXXXXXR)XXXX (SEQ ID NO:26) would allow specific photocleavage at the F* position of the reporter signal.

Multiple photocleavable bonds and/or linkages can be used in or with the same reporter signals or reporter signal conjugates (such as reporter molecules or reporter signal fusions) to achieve a variety of effects. For example, different photocleavable linkages that are cleaved by different wavelengths of light can be used in different parts
5 of reporter signals or reporter signal conjugates to be cleaved at different stages of the method. Different fragmentation wavelengths allow sequential processing which enables, for example, the combinations of the release and fragmentation methods.

As an example, a peptide containing two photocleavable amino acids, Z (cleavage wavelength in the infrared) and F* (photocleavable phenylalanine, cleavage
10 wavelength in UV) can be constructed of the form $XXXXXXXXXXF^*XXXXXXXXR$ where the amino terminus can be attached to an analyte or other molecule utilizing known chemistry. The result is a reporter signal/analyte conjugate (or, alternatively, a reporter molecule). The reporter signal can be released from the conjugate by exposing the conjugate to an appropriate wavelength of light (infrared in this example), thus cleaving
15 the bond at Z. Once the parent ion is selected and stored in the ion trap, the reporter signal can be fragmented by exposing it to an appropriate wavelength of light (UV in this example) to produce the daughter ion ($XXXXXXXXR^+$) which can be detected and quantitated.

Reporter signal calibrators are a special form of reporter signal characterized by
20 their use in reporter signal calibration. Reporter signal calibrators can be any form of reporter signal, as described above and elsewhere herein, but are used as separate molecules that are not physically associated with analytes being assessed. Thus, reporter signal calibrators need not (and preferably do not) have reactive groups for coupling to analytes and need not be (and preferably are not) associated with specific
25 binding molecules or other molecules or components described herein as being associated with reporter signals.

Reporter signal calibrators preferably share one or more common properties with one or more analytes. Reporter signal calibrators and analytes that share one or more common properties are referred to as a reporter signal calibrator/analyte set.
30 When only one analyte and one reporter signal calibrator share the common property they also can be referred to as a reporter signal calibrator/analyte pair. Reporter signal calibrators and analytes in a reporter signal calibrator/analyte set are said to be

matching. The common property allows a reporter signal calibrator and its matching analyte to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signal calibrators and analytes in a set have the same mass-to-charge ratio (m/z). That is, the matching reporter signal calibrators and analytes in a set are isobaric. This allows the reporter signal calibrators and analytes to be separated precisely from other molecules based on mass-to-charge ratio. Reporter signal calibrators can be fragmented, decomposed, reacted, derivatized, or otherwise modified or altered to distinguish the altered reporter signal calibrators from their matching analytes. The analytes can also be fragmented. Preferably, the reporter signal calibrators are fragmented to yield fragments of similar charge but different mass. The reporter signal calibrators can also be fragmented to yield fragments of different charge and mass. Such changes allow the reporter signal calibrator to be distinguished from its matching analyte (and other analytes and/or reporter signal calibrators that are members of the same set, if any) by the different mass-to-charge ratio of the fragment of the reporter signal calibrator. This is possible since, although the unfragmented reporter signal calibrator(s) and analyte(s) in a set are isobaric, the fragments of the reporter signal calibrator(s) are not. Thus, a key feature of the disclosed reporter signal calibrators is that the reporter signal calibrators have a similarity of properties with their matching analytes while the modified reporter signal calibrators are distinguishable from their matching analytes.

Preferred analytes for use with reporter signal calibrators are proteins, peptides, and/or protein fragments (collectively referred to for convenience as proteins). Reporter signal calibrators and proteins that share one or more common properties are referred to as a reporter signal calibrator/protein set. When only one protein and one reporter signal calibrator share the common property they also can be referred to as a reporter signal calibrator/protein pair. Reporter signal calibrators and proteins in a reporter signal calibrator/analyte set are said to be matching.

As described elsewhere herein, reporter signal calibrators can be used as standards for assessing the presence and amount of analytes in samples. For this purpose, a reporter signal calibrator designed for each analyte to be assessed can be mixed with the sample to be analyzed. Analytes and their matching reporter signal calibrators are then processed together to result in detection of both analytes and

reporter signal calibrators (preferably in their altered forms). The amount of reporter signal calibrator or altered reporter signal calibrator detected provides a standard (since the amount of reporter signal calibrator added can be known) against which the amount of analyte or altered analyte detected can be compared. This allows the amount of
5 analyte present in the sample to be accurately gauged.

B. Analytes

The disclosed methods make use of analytes generally as objects of detection, measurement and/or analysis. Analytes can be any molecule or portion of a molecule that is to be detected, measured, or otherwise analyzed. An analyte need not be a
10 physically separate molecule, but may be a part of a larger molecule. Analytes include biological molecules, organic molecules, chemicals, compositions, and any other molecule or structure to which the disclosed method can be adapted. It should be understood that different forms of the disclosed method are more suitable for some types of analytes than other forms of the method. Analytes are also referred to as target
15 molecules.

Preferred analytes are biological molecules. Biological molecules include but are not limited to proteins, peptides, enzymes, amino acid modifications, protein domains, protein motifs, nucleic acid molecules, nucleic acid sequences, DNA, RNA, mRNA, cDNA, metabolites, carbohydrates, and nucleic acid motifs. As used herein,
20 "biological molecule" and "biomolecule" refer to any molecule or portion of a molecule or multi-molecular assembly or composition, that has a biological origin, is related to a molecule or portion of a molecule or multi-molecular assembly or composition that has a biological origin. Biomolecules can be completely artificial molecules that are related to molecules of biological origin.

25 Although reference is made above and elsewhere herein to detection of a "protein" or "proteins," the disclosed method and compositions encompass proteins, peptides, and fragments of proteins or peptides. Thus, reference to a protein herein is intended to refer to proteins, peptides, and fragments of proteins or peptides unless the context clearly indicates otherwise.

C. Analyte Samples

30 Any sample from any source can be used with the disclosed method. In general, analyte samples should be samples that contain, or may contain, analytes. Examples of

suitable analyte samples include cell samples, tissue samples, cell extracts, components or fractions purified from another sample, environmental samples, culture samples, tissue samples, bodily fluids, and biopsy samples. Numerous other sources of samples are known or can be developed and any can be used with the disclosed method.

- 5 Preferred analyte samples for use with the disclosed method are samples of cells and tissues. Analyte samples can be complex, simple, or anywhere in between. For example, an analyte sample may include a complex mixture of biological molecules (a tissue sample, for example), an analyte sample may be a highly purified protein preparation, or a single type of molecule.

10 **D. Protein Samples**

- Any sample from any source can be used with the disclosed method. In general, protein samples should be samples that contain, or may contain, protein molecules. Examples of suitable protein samples include cell samples, tissue samples, cell extracts, components or fractions purified from another sample, environmental samples, biofilm samples, culture samples, tissue samples, bodily fluids, and biopsy samples. Numerous other sources of samples are known or can be developed and any can be used with the disclosed method. Preferred protein samples for use with the disclosed method are samples of cells and tissues. Protein samples can be complex, simple, or anywhere in between. For example, a protein sample may include a complex mixture of proteins (a tissue sample, for example), a protein sample may be a highly purified protein preparation, or a single type of protein.
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E. Reporter Molecules

- Reporter molecules are molecules that combine a reporter signal with a specific binding molecule or decoding tag. Preferably, the reporter signal and specific binding molecule or decoding tag are covalently coupled or tethered to each other. As used herein, molecules are coupled when they are covalent joined, directly or indirectly. One form of indirect coupling is via a linker molecule. The reporter signal can be coupled to the specific binding molecule or decoding tag by any of several established coupling reactions. For example, Hendrickson *et al.*, *Nucleic Acids Res.*, 23(3):522-529 (1995) describes a suitable method for coupling oligonucleotides to antibodies.
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One form of reporter molecule has a peptide nucleic acid as the decoding tag and a reporter signal peptide as the reporter signal. The peptide nucleic acid can

associate with, for example, an oligonucleotide coding tag, thus associating the reporter signal peptide with the coding tag. As described elsewhere herein, coding tags can be used to labeled analytes and other molecules.

As used herein, a molecule is said to be tethered to another molecule when a loop of (or from) one of the molecules passes through a loop of (or from) the other molecule. The two molecules are not covalently coupled when they are tethered. Tethering can be visualized by the analogy of a closed loop of string passing through the hole in the handle of a mug. In general, tethering is designed to allow one or both of the molecules to rotate freely around the loop.

10 F. Specific Binding Molecules

A specific binding molecule is a molecule that interacts specifically with a particular molecule or moiety. The molecule or moiety that interacts specifically with a specific binding molecule is referred to herein as an analyte, such as an analyte.

Preferred analytes are analytes. It is to be understood that the term analyte refers to

15 both separate molecules and to portions of such molecules, such as an epitope of a protein, that interacts specifically with a specific binding molecule. Antibodies, either member of a receptor/ligand pair, synthetic polyamides (Dervan and Burli, *Sequence-specific DNA recognition by polyamides*. Curr Opin Chem Biol, 3(6):688-93 (1999); Wemmer and Dervan, *Targeting the minor groove of DNA*. Curr Opin Struct Biol, 7(3):355-61 (1997)), nucleic acid probes, and other molecules with specific binding
20 affinities are examples of specific binding molecules, useful as the affinity portion of a reporter binding molecule.

A specific binding molecule that interacts specifically with a particular analyte is said to be specific for that analyte. For example, where the specific binding molecule is an antibody that associates with a particular antigen, the specific binding molecule is
25 said to be specific for that antigen. The antigen is the analyte. A reporter molecule containing the specific binding molecule can also be referred to as being specific for a particular analyte. Specific binding molecules preferably are antibodies, ligands, binding proteins, receptor proteins, haptens, aptamers, carbohydrates, synthetic
30 polyamides, peptide nucleic acids, or oligonucleotides. Preferred binding proteins are DNA binding proteins. Preferred DNA binding proteins are zinc finger motifs, leucine

zipper motifs, helix-turn-helix motifs. These motifs can be combined in the same specific binding molecule.

Antibodies useful as the affinity portion of reporter binding agents, can be obtained commercially or produced using well established methods. For example, 5 Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987) on pages 30-85, describe general methods useful for producing both polyclonal and monoclonal antibodies. The entire book describes many general techniques and principles for the use of antibodies in assay systems.

Properties of zinc fingers, zinc finger motifs, and their interactions, are 10 described by Nardelli et al., *Zinc finger-DNA recognition: analysis of base specificity by site-directed mutagenesis*. Nucleic Acids Res, 20(16):4137-44 (1992), Jamieson et al., *In vitro selection of zinc fingers with altered DNA-binding specificity*. Biochemistry, 33(19):5689-95 (1994), Chandrasegaran and Smith, *Chimeric restriction enzymes: what is next?* Biol Chem, 380(7-8):841-8 (1999), and Smith et al., *A detailed 15 study of the substrate specificity of a chimeric restriction enzyme*. Nucleic Acids Res, 27(2):674-81 (1999).

One form of specific binding molecule is an oligonucleotide or oligonucleotide derivative. Such specific binding molecules are designed for and used to detect specific nucleic acid sequences. Thus, the analyte for oligonucleotide specific binding 20 molecules are nucleic acid sequences. The analyte can be a nucleotide sequence within a larger nucleic acid molecule. An oligonucleotide specific binding molecule can be any length that supports specific and stable hybridization between the reporter binding probe and the analyte. For this purpose, a length of 10 to 40 nucleotides is preferred, with an oligonucleotide specific binding molecule 16 to 25 nucleotides long being most 25 preferred. It is preferred that the oligonucleotide specific binding molecule is peptide nucleic acid. Peptide nucleic acid forms a stable hybrid with DNA. This allows a peptide nucleic acid specific binding molecule to remain firmly adhered to the target sequence during subsequent amplification and detection operations.

This useful effect can also be obtained with oligonucleotide specific binding 30 molecules by making use of the triple helix chemical bonding technology described by Gasparro et al., *Nucleic Acids Res.*, 22(14):2845-2852 (1994). Briefly, the oligonucleotide specific binding molecule is designed to form a triple helix when

hybridized to a target sequence. This is accomplished generally as known, preferably by selecting either a primarily homopurine or primarily homopyrimidine target sequence. The matching oligonucleotide sequence which constitutes the specific binding molecule will be complementary to the selected target sequence and thus be primarily homopyrimidine or primarily homopurine, respectively. The specific binding molecule (corresponding to the triple helix probe described by Gasparro *et al.*) contains a chemically linked psoralen derivative. Upon hybridization of the specific binding molecule to a target sequence, a triple helix forms. By exposing the triple helix to low wavelength ultraviolet radiation, the psoralen derivative mediates cross-linking of the probe to the target sequence.

G. Reporter Signal Fusions

Reporter signal fusions are reporter signal peptides joined with a protein or peptide of interest in a single amino acid segment (that is, a fusion protein). Such fusions of proteins and peptides of interest with reporter signal peptides can be expressed as a fusion protein or peptide from a nucleic acid molecule encoding the amino acid segment that constitutes the fusion. A reporter signal fusion nucleic acid molecule or reporter signal nucleic acid segment refers to a nucleic acid molecule or nucleic acid sequence, respectively, that encodes a reporter signal fusion. Although reference is made above and elsewhere herein to detection of, and fusion with, a "protein" or "proteins," the disclosed reporter signal fusions encompass fusions with proteins, peptides, and fragments of proteins or peptides. Thus, reference to a protein herein is intended to refer to proteins, peptides, and fragments of proteins or peptides unless the context clearly indicates otherwise.

As used herein "reporter signal fusion" refers to a protein, peptide, or fragment of a protein or peptide to which a reporter signal peptide is fused (that is, joined by peptide bond(s) in the same polypeptide chain) unless the context clearly indicates otherwise. The reporter signal peptide and the protein of interest involved in a reporter signal fusion need not be directly fused. That is, other amino acids, amino acid sequences, and/or peptide elements can intervene. For example, an epitope tag, if present, can be located between the protein of interest and the reporter signal peptide in a reporter signal fusion. The reporter signal peptide(s) can be fused to a protein in any arrangement, such as at the N-terminal end of the protein, at the C-terminal end of the

protein, in or at domain junctions, or at any other appropriate location in the protein. In some forms of the method, it is desirable that the protein remain functional. In such cases, terminal fusions or inter-domain fusions are preferably. Those of skill in the art of protein fusions generally know how to design fusions where the protein of interest
5 remains functional. In other embodiments, it is not necessary that the protein remain functional in which case the reporter signal peptide and protein can have any desired structural organization.

A given reporter signal fusion can include one or more reporter signal peptides and one or more proteins or peptides of interest. In addition, reporter signal fusions can
10 include one or more amino acids, amino acid sequences, and/or peptide elements. The disclosed reporter signal fusions comprise a single, contiguous polypeptide chain. Thus, although multiple amino acid segments can be part of the same contiguous polypeptide chain, all of the components (that is, the reporter signal peptide(s) and protein(s) and peptide(s) of interest) of a given amino acid segment are part of the same
15 contiguous polypeptide chain.

Reporter signal fusions can be produced by expression from nucleic acid molecules encoding the fusions. Thus, the disclosed fusions generally can be designed by designing nucleic acid segments that encode amino acid segments where the amino acid segments comprise a reporter signal peptide and a protein or peptide of interest. A
20 given nucleic acid molecule can comprise one or more nucleic acid segments. A given nucleic acid segment can encode one or more amino acid segments. A given amino acid segment can include one or more reporter signal peptides and one or more proteins or peptides of interest. The disclosed amino acid segments consist of a single, contiguous polypeptide chain. Thus, although multiple amino acid segments can be
25 part of the same contiguous polypeptide chain, all of the components (that is, the reporter signal peptide(s) and protein(s) and peptide(s) of interest) of a given amino acid segment are part of the same contiguous polypeptide chain.

Reporter signal fusions can include other components besides a protein of interest and a reporter signal peptide. For example, reporter signal fusions can include
30 epitope tags or flag peptides (see, for example, Groth et al. (2000) A phage integrase directs efficient site-specific integration in human cells. Proc Natl Acad Sci U S A 97:5995-6000). Epitope tags and flag peptides can serve as tags by which reporter

signal fusions can be separated, distinguished, associated, and/or bound. The use of epitope tags and flag peptides generally is known and can be adapted for use in the disclosed reporter signal fusions.

Reporter signal peptides can be fragmented, decomposed, reacted, derivatized, or otherwise modified, preferably in a characteristic way. This allows a protein to which the reporter signal peptide is fused to be identified by detection of one or more of the products of the reporter signal fusion following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal peptide. The protein can also be identified by the correlated detection of the reporter signal fusion and one or more of the products of the reporter signal fusion following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal peptide. The alteration of the reporter signal peptide will alter the reporter signal fusion in a characteristic and detectable way. Together, the detection of a characteristic reporter signal fusion and a characteristic product of (that is, altered form of) the reporter signal fusion can uniquely identify the protein (although the altered form alone can be detected, if desired). In this way, expression of one or more proteins can be detected, either alone or together (for example, in a multiplex assay). Further, expression of one or more proteins in one or more samples can be detected in a multiplex manner. Preferably, for mass spectrometry reporter signals, the reporter signal peptides are fragmented to yield fragments of similar charge but different mass.

Preferably, the reporter signal peptides are designed to be fragmented to yield fragments of similar charge but different mass. This allows each reporter signal fusion (and/or each reporter signal peptide) in a set to be distinguished by the different mass-to-charge ratios of the fragments of (that is, altered forms of) the reporter signal peptides. This is possible since the fragments of the different reporter signal peptides (or the fragments of the reporter signal fusions) can be designed to have different mass-to-charge ratios. In the disclosed method, this allows each reporter signal fusion to be distinguished by the mass-to-charge ratios of the reporter signal fusions after fragmentation of the reporter signal peptide.

Alteration of reporter signal peptides in reporter signal fusions can produce a variety of altered compositions. Any or all of these altered forms can be detected. For example, the altered form of the reporter signal peptide can be detected, the altered

form of the amino acid segment (which contains the reporter signal peptide) can be detected, the altered form of a subsegment of the amino acid segment can be detected, or a combination of these can be detected. Where the reporter signal peptide is altered by fragmentation, the result generally will be a fragment of the reporter signal peptide and an altered form of the amino acid segment containing the protein or peptide of interest and a portion of the reporter signal peptide (that is, the portion not in the reporter signal peptide fragment).

The protein or peptide of interest also can be fragmented. The result would be a subsegment of the amino acid segment. The amino acid subsegment would contain the reporter signal peptide and a portion of the protein or peptide of interest. When the reporter signal peptide in an amino acid subsegment is altered (which can occur before, during, or after fragmentation of the amino acid segment), the result is an altered form of the amino acid subsegment (and an altered form of the reporter signal peptide). This altered form of amino acid subsegment can be detected. Where the reporter signal peptide is altered by fragmentation, the result generally will be a fragment of the reporter signal peptide and an altered form of (that is, fragment of) the amino acid subsegment. In this case, the altered form of the amino acid subsegment, which is also referred to herein as a reporter signal fusion fragment, will contain a portion of the protein or peptide of interest and a portion of the reporter signal peptide (that is, the portion not in the reporter signal peptide fragment).

As with reporter signals generally, reporter signal fusions (also referred to as amino acid segments), reporter signal fusion fragments (also referred to as subsegments of the reporter signal fusions), or reporter signal peptides can be used in sets where the reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides in a set can have one or more common properties that allow the reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides to be separated or distinguished from molecules lacking the common property. In the case of reporter signal fusions, amino acid segments and amino acid subsegments can be used in sets where the amino acid segments and amino acid subsegments in a set can have one or more common properties that allow the amino acid segments and amino acid subsegments, respectively, to be separated or distinguished from molecules lacking the common property. In general, the component(s) of the reporter signal fusions having

common properties can depend on the component(s) to be detected and/or the mode of the method being used.

A variety of different properties can be used as the common physical property used to separate reporter signal fusions, reporter signal fusion fragments, and/or reporter signal peptides from other molecules lacking the common property. For example, physical properties useful as common properties include mass-to-charge ratio, mass, charge, isoelectric point, hydrophobicity, chromatography characteristics, and density. It is preferred that the physical property shared by reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides in a set (and used to distinguish or separate the reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides from other molecules) is an overall property of the reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides (for example, overall mass, overall charge, isoelectric point, overall hydrophobicity, etc.) rather than the mere presence of a feature or moiety (for example, an affinity tag, such as biotin). Such properties are referred to herein as "overall" properties (and thus, reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides in a set would be referred to as sharing a "common overall property"). It should be understood that reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides can have features and moieties, such as affinity tags, and that such features and moieties can contribute to the common overall property (by contributing mass, for example). However, such limited and isolated features and moieties would not serve as the sole basis of the common overall property.

It is preferred that the common property of reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides is not an affinity tag. Nevertheless, even in such a case, reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides that otherwise have a common property may also include an affinity tag—and in fact may all share the same affinity tag—so long as another common property is present that can be (and, in some embodiments of the disclosed method, is) used to separate reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides sharing the common property from other molecules lacking the common property. With this in mind, it is preferred that, if chromatography or other separation techniques are used to separate reporter signal fusions, reporter signal fusion

fragments, or reporter signal peptides based on the common property, the affinity be based on an overall physical property of the reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides and not on the presence of, for example, a feature or moiety such as an affinity tag. As used herein, a common property is a
5 property shared by a set of components (such as reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides). That is, the components have the property "in common." It should be understood that reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides in a set may have numerous properties in common. However, as used herein, the common properties of reporter
10 signal fusions, reporter signal fusion fragments, or reporter signal peptides referred to are only those used in the disclosed method to distinguish and/or separate the reporter signal fusions, reporter signal fusion fragments, or reporter signal peptides sharing the common property from molecules that lack the common property.

In preferred embodiments, reporter signal peptides, reporter signal fusions (or
15 amino acid segments), nucleic acid segments encoding reporter signal fusions, and/or nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions are used in sets where the reporter signal peptides, the reporter signal fusions, and/or subsegments of the reporter signal fusions constituting or present in the set have similar properties (such as similar mass-to-charge ratios). The similar properties allow
20 the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions constituting or present in a set have the same mass-to-charge ratio (m/z). That is, the reporter signals, the reporter signal fusions, or
25 subsegments of the reporter signal fusions in a set are isobaric. This allows the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions to be separated precisely from other molecules based on mass-to-charge ratio. The result of the filtering is a huge increase in the signal to noise ratio (S/N) for the system, allowing more sensitive and accurate detection.

30 Sets of reporter signal fusions (also referred to as amino acid segments), reporter signal fusion fragments (also referred to as subsegments of the reporter signal fusions or amino acid subsegments), reporter signal peptides, nucleic acid segments

encoding reporter signal fusions, or nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions can have any number of reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, or nucleic acid molecules comprising
5 nucleic acid segments encoding reporter signal fusions. For example, sets of reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, or nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions can have one, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more,
10 nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, one hundred or more, two hundred or more, three hundred or more, four hundred or more, or five hundred or more different reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, or nucleic acid
15 molecules comprising nucleic acid segments encoding reporter signal fusions. Although specific numbers of reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, and nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions, and specific endpoints for ranges of the number of reporter signal fusions,
20 reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, and nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions, are recited, each and every specific number of reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, and nucleic acid molecules
25 comprising nucleic acid segments encoding reporter signal fusions, and each and every specific endpoint of ranges of numbers of reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, and nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions, are specifically contemplated, although not explicitly listed, and
30 each and every specific number of reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, and nucleic acid molecules comprising nucleic acid segments encoding

reporter signal fusions, and each and every specific endpoint of ranges of numbers of reporter signal fusions, reporter signal fusion fragments, reporter signal peptides, nucleic acid segments encoding reporter signal fusions, and nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions, are hereby
5 specifically described.

The reporter signal fusions are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge ratios. A set of isobaric reporter signal peptides or reporter signal fusions can be used for multiplex labeling and/or detection of the expression of many genes, proteins,
10 vectors, expression constructs, cells, cell lines, and organisms since the reporter signal peptide fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection. Where the same gene, protein, vectors, expression construct, cell, cell line, or organism (or the same type of gene, protein, vector, expression construct, cell, cell line, or organism) is labeled with a set of reporter
15 signal fusions that are isobaric or that include isobaric reporter signal peptides (by, for example, "labeling" the same gene, protein, vector, expression construct, cell, cell line, or organism in different samples), the set of reporter signal fusions or reporter signal peptides that results will also be isobaric. Fragmentation of the reporter signal peptides will split the set of reporter signal peptides into individually detectable reporter signal
20 fusion fragments and reporter signal peptide fragments of characteristically different mass.

Reporter signal fusions can be expressed in any suitable manner. For example, nucleic acid sequences and nucleic acid segments encoding reporter signal fusions can be expressed in vitro, in cells, and/or in cells in organism. Many techniques and
25 systems for expression of nucleic acid sequences and proteins are known and can be used with the disclosed reporter signal fusions. For example, many expression sequences, vector systems, transformation and transfection techniques, and transgenic organism production methods are known and can be used with the disclosed reporter signal peptide method and compositions. Systems are known for integration of nucleic
30 acid constructs into chromosomes of cells and organisms (see, for example, Groth et al. (2000) A phage integrase directs efficient site-specific integration in human cells. Proc Natl Acad Sci U S A 97:5995-6000; Hong et al. (2001) Development of two bacterial

artificial chromosome shuttle vectors for a recombination-based cloning and regulated expression of large genes in mammalian cells. Analytical Biochemistry 291:142-148) which can be used with the disclosed nucleic acid molecules and segments encoding reporter signal fusions or to form nucleic acid segment encoding reporter signal fusions.

As used herein, an expression sample is a sample that contains, or might contain, one or more reporter signal fusions expressed from a nucleic acid molecule. An expression sample to be analyzed can be subjected to fractionation or separation to reduce the complexity of the samples. Fragmentation and fractionation can also be used together in the same assay. Such fragmentation and fractionation can simplify and extend the analysis of the expression.

Nucleic acid molecules encoding reporter signal fusions can be used in sets where the reporter signal peptides in the reporter signal fusions encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Similarly, nucleic acid molecules encoding amino acid segments can be used in sets where the reporter signal peptides in the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Nucleic acid molecules encoding amino acid segments can be used in sets where the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the amino acid segments to be separated or distinguished from molecules lacking the common property.

Nucleic acid segments (which, generally, are part of nucleic acid molecules) encoding reporter signal fusions can be used in sets where the reporter signal peptides in the reporter signal fusions encoded by a set of nucleic acid segments can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Similarly, nucleic acid segments encoding amino acid segments can be used in sets where the reporter signal peptides in the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Nucleic acid

segments encoding amino acid segments can be used in sets where the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the amino acid segments to be separated or distinguished from molecules lacking the common property. Other relationships between members of the sets of nucleic acid molecules, nucleic acid segments, amino acid segments, reporter signal peptides, and proteins of interest are contemplated.

Reporter signal fusions allow sensitive and multiplex detection of expression of particular proteins and peptides of interest, and/or of the genes, vectors, and expression constructs encoding the proteins and peptides of interest. The disclosed reporter signal fusions can also be used for any purpose including as a source of reporter signals for other forms of the disclosed method and compositions.

H. Reporter Signal/Analyte Conjugates

Compositions where reporter signals are associated with, incorporated into, or otherwise linked to the analytes are referred to as reporter signal/analyte conjugates. Such conjugates include reporter signals associated with analytes, such as a reporter signal probe hybridized to a nucleic acid sequence; reporter signals covalently coupled to analytes, such as reporter signals linked to proteins via a linking group; and reporter signals incorporated into analytes, such as fusions between a protein of interest and a peptide reporter signal.

Reporter signal/analyte conjugates can be altered, generally through alteration of the reporter signal portion of the conjugate, such that the altered forms of different reporter signals, altered forms of different reporter signal/analyte conjugates, or both, can be distinguished from each other. Where the reporter signal or reporter signal/analyte conjugate is altered by fragmentation, any, some, or all of the fragments can be distinguished from each other, depending on the embodiment. For example, where reporter signal/analyte conjugates are fragmented into two parts (with the break point in the reporter signal portion), either the reporter signal fragment, the reporter signal/analyte fragment, or both can be distinguished.

Sets of reporter signal/analyte conjugates can be used where two or more of the reporter signal/analyte conjugates in a set have one or more common properties that allow the reporter signal/analyte conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. In

still other embodiments, analytes can be fragmented (prior to or following conjugation) to produce reporter signal/analyte fragment conjugates (which can be referred to as fragment conjugates). In such cases, sets of fragment conjugates can be used where two or more of the fragment conjugates in a set have one or more common properties that allow the fragment conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. It should be understood that fragmented analytes can be considered analytes in their own right. In this light, reference to fragmented analytes is made for convenience and clarity in describing certain embodiments and to allow reference to both the base analyte and the fragmented analyte.

Sets of reporter signal/analyte conjugates or reporter signal/analyte fragment conjugates (fragment conjugates) can have any number of reporter signal/analyte conjugates or reporter signal/analyte fragment conjugates. For example, sets of reporter signal/analyte conjugates or reporter signal/analyte fragment conjugates can have one, two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, one hundred or more, two hundred or more, three hundred or more, four hundred or more, or five hundred or more different reporter signal/analyte conjugates or reporter signal/analyte fragment conjugates. Although specific numbers of reporter signal/analyte conjugates and reporter signal/analyte fragment conjugates, and specific endpoints for ranges of the number of reporter signal/analyte conjugates and reporter signal/analyte fragment conjugates, are recited, each and every specific number of reporter signal/analyte conjugates and reporter signal/analyte fragment conjugates, and each and every specific endpoint of ranges of numbers of reporter signal/analyte conjugates and reporter signal/analyte fragment conjugates, are specifically contemplated, although not explicitly listed, and each and every specific number of reporter signal/analyte conjugates and reporter signal/analyte fragment conjugates, and each and every specific endpoint of ranges of numbers of reporter signal/analyte conjugates and reporter signal/analyte fragment conjugates, are hereby specifically described.

As indicated above, reporter signals conjugated with analytes can be altered while in the conjugate and distinguished. Conjugated reporter signals can also be dissociated or separated, in whole or in part, from the conjugated analytes prior to their alteration. Where the reporter signals are dissociated (in whole or in part) from the analytes, the method can be performed such that the fact of association between the analyte and reporter signal is part of the information obtained when the reporter signal is detected. In other words, the fact that the reporter signal may be dissociated from the analyte for detection does not obscure the information that the detected reporter signal was associated with the analyte.

As used herein, reporter signal conjugate refers both to reporter signal/analyte conjugates and to other components of the disclosed method such as reporter molecules.

As with reporter signals generally, reporter signal/analyte conjugates and reporter signal/analyte fragment conjugates can be used in sets where the reporter signal/analyte conjugates or fragment conjugates in a set can have one or more common properties that allow the reporter signal/analyte conjugates or fragment conjugates to be separated or distinguished from molecules lacking the common property. As with reporter signals generally, a variety of different properties can be used as the common physical property used to separate reporter signal/analyte conjugates or fragment conjugates from other molecules lacking the common property. For example, physical properties useful as common properties include mass-to-charge ratio, mass, charge, isoelectric point, hydrophobicity, chromatography characteristics, and density. It is preferred that the physical property shared by reporter signal/analyte conjugates or fragment conjugates in a set (and used to distinguish or separate the reporter signal/analyte conjugates or fragment conjugates from other molecules) is an overall property of the reporter signal/analyte conjugates or fragment conjugates (for example, overall mass, overall charge, isoelectric point, overall hydrophobicity, etc.) rather than the mere presence of a feature or moiety (for example, an affinity tag, such as biotin). Such properties are referred to herein as "overall" properties (and thus, reporter signal/analyte conjugates or fragment conjugates in a set would be referred to as sharing a "common overall property"). It should be understood that reporter signal/analyte conjugates or fragment conjugates can have features and moieties, such

as affinity tags, and that such features and moieties can contribute to the common overall property (by contributing mass, for example). However, such limited and isolated features and moieties would not serve as the sole basis of the common overall property.

5 It is preferred that the common property of reporter signal/analyte conjugates or fragment conjugates is not an affinity tag. Nevertheless, even in such a case, reporter signal/analyte conjugates or fragment conjugates that otherwise have a common property may also include an affinity tag—and in fact may all share the same affinity tag—so long as another common property is present that can be (and, in some
10 embodiments of the disclosed method, is) used to separate reporter signal/analyte conjugates or fragment conjugates sharing the common property from other molecules lacking the common property. With this in mind, it is preferred that, if chromatography or other separation techniques are used to separate reporter signal/analyte conjugates or fragment conjugates based on the common property, the affinity be based on an overall
15 physical property of the reporter signal/analyte conjugates or fragment conjugates and not on the presence of, for example, a feature or moiety such as an affinity tag. As used herein, a common property is a property shared by a set of components (such as reporter signal/analyte conjugates or fragment conjugates). That is, the components have the property "in common." It should be understood that reporter signal/analyte
20 conjugates or fragment conjugates in a set may have numerous properties in common. However, as used herein, the common properties of reporter signal/analyte conjugates or fragment conjugates referred to are only those used in the disclosed method to distinguish and/or separate the reporter signal/analyte conjugates or fragment conjugates sharing the common property from molecules that lack the common
25 property.

I. Capture Arrays

 A capture array (also referred to herein as an array) includes a plurality of capture tags immobilized on a solid-state substrate, preferably at identified or predetermined locations on the solid-state substrate. In this context, plurality of capture
30 tags refers to a multiple capture tags each having a different structure. Preferably, each predetermined location on the array (referred to herein as an array element) has one type of capture tag (that is, all the capture tags at that location have the same structure).

Each location will have multiple copies of the capture tag. The spatial separation of capture tags of different structure in the array allows separate detection and identification of analytes that become associated with the capture tags. If a decoding tag is detected at a given location in a capture array, it indicates that the analyte
5 corresponding to that array element was present in the target sample.

Solid-state substrates for use in capture arrays can include any solid material to which capture tags can be coupled, directly or indirectly. This includes materials such as acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass,
10 polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin films or membranes, beads, bottles, dishes, disks, compact disks, fibers, optical fibers, woven fibers, shaped polymers,
15 particles and microparticles. A preferred form for a solid-state substrate is a compact disk.

Although preferred, it is not required that a given capture array be a single unit or structure. The set of capture tags may be distributed over any number of solid supports. For example, at one extreme, each capture tag may be immobilized in a
20 separate reaction tube or container. Arrays may be constructed upon non permeable or permeable supports of a wide variety of support compositions such as those described above. The array spot sizes and density of spot packing vary over a tremendous range depending upon the process(es) and material(s) used.

Methods for immobilizing antibodies and other proteins to substrates are well
25 established. Immobilization can be accomplished by attachment, for example, to aminated surfaces, carboxylated surfaces or hydroxylated surfaces using standard immobilization chemistries. Examples of attachment agents are cyanogen bromide, succinimide, aldehydes, tosyl chloride, avidin-biotin, photocrosslinkable agents, epoxides and maleimides. A preferred attachment agent is glutaraldehyde. These and
30 other attachment agents, as well as methods for their use in attachment, are described in *Protein immobilization: fundamentals and applications*, Richard F. Taylor, ed. (M. Dekker, New York, 1991), Johnstone and Thorpe, *Immunochemistry In Practice*

(Blackwell Scientific Publications, Oxford, England, 1987) pages 209-216 and 241-242, and *Immobilized Affinity Ligands*, Craig T. Hermanson *et al.*, eds. (Academic Press, New York, 1992). Antibodies can be attached to a substrate by chemically cross-linking a free amino group on the antibody to reactive side groups present within the substrate. For example, antibodies may be chemically cross-linked to a substrate that contains free amino or carboxyl groups using glutaraldehyde or carbodiimides as cross-linker agents. In this method, aqueous solutions containing free antibodies are incubated with the solid-state substrate in the presence of glutaraldehyde or carbodiimide. For crosslinking with glutaraldehyde the reactants can be incubated with 2% glutaraldehyde by volume in a buffered solution such as 0.1 M sodium cacodylate at pH 7.4. Other standard immobilization chemistries are known by those of skill in the art.

Methods for immobilization of oligonucleotides to solid-state substrates are well established. Oligonucleotide capture tags can be coupled to substrates using established coupling methods. For example, suitable attachment methods are described by Pease *et al.*, *Proc. Natl. Acad. Sci. USA* 91(11):5022-5026 (1994), Khrapko *et al.*, *Mol Biol (Mosk) (USSR)* 25:718-730 (1991), U.S. Patent No. 5,871,928 to Fodor *et al.*, U.S. Patent No. 5,654,413 to Brenner, U.S. Patent No. 5,429,807, and U.S. Patent No. 5,599,695 to Pease *et al.* A method for immobilization of 3'-amine oligonucleotides on casein-coated slides is described by Stimpson *et al.*, *Proc. Natl. Acad. Sci. USA* 92:6379-6383 (1995). A preferred method of attaching oligonucleotides to solid-state substrates is described by Guo *et al.*, *Nucleic Acids Res.* 22:5456-5465 (1994).

Planar array technology has been utilized for many years (Shalon, D., S.J. Smith, and P.O. Brown, A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Res*, 1996. 6(7): p. 639-45, Singh-Gasson, S., *et al.*, Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array. *Nat Biotechnol*, 1999. 17(10): p. 974-8, Southern, E.M., U. Maskos, and J.K. Elder, Analyzing and comparing nucleic acid sequences by hybridization to arrays of oligonucleotides: evaluation using experimental models. *Genomics*, 1992. 13(4): p. 1008-17, Nizetic, D., *et al.*, Construction, arraying, and high-density screening of large insert libraries of human chromosomes X and 21: their potential use as reference libraries. *Proc Natl Acad Sci*

- U S A, 1991. 88(8): p. 3233-7, Van Oss, C.J., R.J. Good, and M.K. Chaudhury, *Mechanism of DNA (Southern) and protein (Western) blotting on cellulose nitrate and other membranes*. J Chromatogr, 1987. 391(1): p. 53-65, Ramsay, G., *DNA chips: state-of-the art*. Nat Biotechnol, 1998. 16(1): p. 40-4, Schena, M., *et al.*, *Parallel human genome analysis: microarray-based expression monitoring of 1000 genes*. Proc Natl Acad Sci U S A, 1996. 93(20): p. 10614-9, Lipshutz, R.J., *et al.*, *High density synthetic oligonucleotide arrays*. Nat Genet, 1999. 21(1 Suppl): p. 20-4, Pease, A.C., *et al.*, *Light-generated oligonucleotide arrays for rapid DNA sequence analysis*. Proc Natl Acad Sci U S A, 1994. 91(11): p. 5022-6, Maier, E., *et al.*, *Application of robotic technology to automated sequence fingerprint analysis by oligonucleotide hybridisation*. J Biotechnol, 1994. 35(2-3): p. 191-203, Vasiliskov, A.V., *et al.*, *Fabrication of microarray of gel-immobilized compounds on a chip by copolymerization*. Biotechniques, 1999. 27(3): p. 592-4, 596-8, 600 passim, and Yershov, G., *et al.*, *DNA analysis and diagnostics on oligonucleotide microchips*. Proc Natl Acad Sci U S A, 1996. 93(10): p. 4913-8).

Oligonucleotide capture tags in arrays can also be designed to have similar hybrid stability. This would make hybridization of fragments to such capture tags more efficient and reduce the incidence of mismatch hybridization. The hybrid stability of oligonucleotide capture tags can be calculated using known formulas and principles of thermodynamics (see, for example, Santa Lucia *et al.*, *Biochemistry* 35:3555-3562 (1996); Freier *et al.*, *Proc. Natl. Acad. Sci. USA* 83:9373-9377 (1986); Breslauer *et al.*, *Proc. Natl. Acad. Sci. USA* 83:3746-3750 (1986)). The hybrid stability of the oligonucleotide capture tags can be made more similar (a process that can be referred to as smoothing the hybrid stabilities) by, for example, chemically modifying the capture tags (Nguyen *et al.*, *Nucleic Acids Res.* 25(15):3059-3065 (1997); Hohsisel, *Nucleic Acids Res.* 24(3):430-432 (1996)). Hybrid stability can also be smoothed by carrying out the hybridization under specialized conditions (Nguyen *et al.*, *Nucleic Acids Res.* 27(6):1492-1498 (1999); Wood *et al.*, *Proc. Natl. Acad. Sci. USA* 82(6):1585-1588 (1985)).

Another means of smoothing hybrid stability of the oligonucleotide capture tags is to vary the length of the capture tags. This would allow adjustment of the hybrid stability of each capture tag so that all of the capture tags had similar hybrid stabilities

(to the extent possible). Since the addition or deletion of a single nucleotide from a capture tag will change the hybrid stability of the capture tag by a fixed increment, it is understood that the hybrid stabilities of the capture tags in a capture array will not be equal. For this reason, similarity of hybrid stability as used herein refers to any
5 increase in the similarity of the hybrid stabilities of the capture tags (or, put another way, any reduction in the differences in hybrid stabilities of the capture tags).

The efficiency of hybridization and ligation of oligonucleotide capture tags to sample fragments can also be improved by grouping capture tags of similar hybrid stability in sections or segments of a capture array that can be subjected to different
10 hybridization conditions. In this way, the hybridization conditions can be optimized for particular classes of capture tags.

J. Capture Tags

A capture tag is any compound that can be used to capture or separate compounds or complexes having the capture tag. Preferably, a capture tag is a
15 compound that interacts specifically with a particular molecule or moiety. Preferably, the molecule or moiety that interacts specifically with a capture tag is an analyte. It is to be understood that the term analyte refers to both separate molecules and to portions of such molecules, such as an epitope of a protein, that interacts specifically with a capture tag. Antibodies, either member of a receptor/ligand pair, synthetic polyamides
20 (Dervan and Burli, *Sequence-specific DNA recognition by polyamides*. Curr Opin Chem Biol, 3(6):688-93 (1999); Wemmer and Dervan, *Targeting the minor groove of DNA*. Curr Opin Struct Biol, 7(3):355-61 (1997)), nucleic acid probes, and other molecules with specific binding affinities are examples of capture tags.

A capture tag that interacts specifically with a particular analyte is said to be
25 specific for that analyte. For example, where the capture tag is an antibody that associates with a particular antigen, the capture tag is said to be specific for that antigen. The antigen is the analyte. Capture tags preferably are antibodies, ligands, binding proteins, receptor proteins, haptens, aptamers, carbohydrates, synthetic polyamides, peptide nucleic acids, or oligonucleotides. Preferred binding proteins are
30 DNA binding proteins. Preferred DNA binding proteins are zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs. These motifs can be combined in the same capture tag.

Antibodies useful as the affinity portion of reporter binding agents, can be obtained commercially or produced using well established methods. For example, Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987) on pages 30-85, describe general methods useful for producing both polyclonal and monoclonal antibodies. The entire book describes many general techniques and principles for the use of antibodies in assay systems.

Properties of zinc fingers, zinc finger motifs, and their interactions, are described by Nardelli et al., *Zinc finger-DNA recognition: analysis of base specificity by site-directed mutagenesis*. *Nucleic Acids Res*, 20(16):4137-44 (1992), Jamieson et al., *In vitro selection of zinc fingers with altered DNA-binding specificity*. *Biochemistry*, 33(19):5689-95 (1994), Chandrasegaran and Smith, *Chimeric restriction enzymes: what is next?* *Biol Chem*, 380(7-8):841-8 (1999), and Smith et al., *A detailed study of the substrate specificity of a chimeric restriction enzyme*. *Nucleic Acids Res*, 27(2):674-81 (1999).

One form of capture tag is an oligonucleotide or oligonucleotide derivative. Such capture tags are designed for and used to detect specific nucleic acid sequences. Thus, the analyte for oligonucleotide capture tags are nucleic acid sequences. The analyte can be a nucleotide sequence within a larger nucleic acid molecule. An oligonucleotide capture tag can be any length that supports specific and stable hybridization between the capture tag and the analyte. For this purpose, a length of 10 to 40 nucleotides is preferred, with an oligonucleotide capture tag 16 to 25 nucleotides long being most preferred. It is preferred that the oligonucleotide capture tag is peptide nucleic acid. Peptide nucleic acid forms a stable hybrid with DNA. This allows a peptide nucleic acid capture tag to remain firmly adhered to the target sequence during subsequent amplification and detection operations.

This useful effect can also be obtained with oligonucleotide capture tags by making use of the triple helix chemical bonding technology described by Gasparro et al., *Nucleic Acids Res.*, 22(14):2845-2852 (1994). Briefly, the oligonucleotide capture tag is designed to form a triple helix when hybridized to a target sequence. This is accomplished generally as known, preferably by selecting either a primarily homopurine or primarily homopyrimidine target sequence. The matching oligonucleotide sequence which constitutes the capture tag will be complementary to

the selected target sequence and thus be primarily homopyrimidine or primarily homopurine, respectively. The capture tag (corresponding to the triple helix probe described by Gasparro *et al.*) contains a chemically linked psoralen derivative. Upon hybridization of the capture tag to a target sequence, a triple helix forms. By exposing the triple helix to low wavelength ultraviolet radiation, the psoralen derivative mediates cross-linking of the probe to the target sequence.

K. Sample Arrays

A sample array includes a plurality of samples (for example, expression samples, tissue samples, protein samples) immobilized on a solid-state substrate, preferably at identified or predetermined locations on the solid-state substrate. Preferably, each predetermined location on the sample array (referred to herein as a sample array element) has one type of sample. The spatial separation of different samples in the sample array allows separate detection and identification of reporter signals (or reporter molecules or coding tags) that become associated with the samples. If a reporter signal is detected at a given location in a sample array, it indicates that the analyte corresponding to that reporter signal was present in the sample corresponding to that sample array element.

Solid-state substrates for use in sample arrays can include any solid material to which samples can be adhered, directly or indirectly. This includes materials such as acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin films or membranes, beads, bottles, dishes, disks, compact disks, fibers, optical fibers, woven fibers, shaped polymers, particles and microparticles. A preferred form for a solid-state substrate is a compact disk.

Although preferred, it is not required that a given sample array be a single unit or structure. The set of samples may be distributed over any number of solid supports. For example, at one extreme, each sample may be immobilized in a separate reaction tube or container. Sample arrays may be constructed upon non permeable or permeable

supports of a wide variety of support compositions such as those described above. The array spot sizes and density of spot packing vary over a tremendous range depending upon the process(es) and material(s) used. Methods for adhering or immobilizing samples and sample components to substrates are well established.

- 5 A preferred form of sample array is a tissue arrays, where there are small tissue samples on a substrate. Such tissue microarrays exist, and are used, for example, in a cohort to study breast cancer. The disclosed method can be used, for example, to probe multiple analytes in multiple samples. Sample arrays can be, for example, labeled with different reporter signals, the whole support then introduced into source region of a
10 mass spec, and sampled by MALDI.

L. Decoding Tags

- Decoding tags are any molecule or moiety that can be associated with coding tags, directly or indirectly. Decoding tags are associated with reporter signals (making up a reporter molecule) to allow indirect association of the reporter signals with an
15 analyte. Decoding tags preferably are oligonucleotides, carbohydrates, synthetic polyamides, peptide nucleic acids, antibodies, ligands, proteins, haptens, zinc fingers, aptamers, or mass labels.

- Preferred decoding tags are molecules capable of hybridizing specifically to an oligonucleotide coding tag. Most preferred are peptide nucleic acid decoding tags.
20 Oligonucleotide or peptide nucleic acid decoding tags can have any arbitrary sequence. The only requirement is hybridization to coding tags. The decoding tags can each be any length that supports specific and stable hybridization between the coding tags and the decoding tags. For this purpose, a length of 10 to 35 nucleotides is preferred, with a decoding tag 15 to 20 nucleotides long being most preferred.

- 25 Reporter molecules containing decoding tags preferably are capable of being released by matrix-assisted laser desorption-ionization (MALDI) in order to be separated and identified by time-of-flight (TOF) mass spectroscopy, or by another detection technique. A decoding tag may be any oligomeric molecule that can hybridize to a coding tag. For example, a decoding tag can be a DNA oligonucleotide,
30 an RNA oligonucleotide, or a peptide nucleic acid (PNA) molecule. Preferred decoding tags are PNA molecules.

M. Coding Tags

Coding tags are molecules or moieties with which decoding tags can associate. Coding tags can be any type of molecule or moiety that can serve as a target for decoding tag association. Preferred coding tags are oligomers, oligonucleotides, or nucleic acid sequences. Coding tags can also be a member of a binding pair, such as streptavidin or biotin, where its cognate decoding tag is the other member of the binding pair. Coding tags can also be designed to associate directly with some types of reporter signals. For example, oligonucleotide coding tags can be designed to interact directly with peptide nucleic acid reporter signals (which are reporter signals composed of peptide nucleic acid).

The oligomeric base sequences of oligomeric coding tags can include RNA, DNA, modified RNA or DNA, modified backbone nucleotide-like oligomers such as peptide nucleic acid, methylphosphonate DNA, and 2'-O-methyl RNA or DNA. Oligomeric or oligonucleotide coding tags can have any arbitrary sequence. The only requirement is association with decoding tags (preferably by hybridization). In the disclosed method, multiple coding tags can become associated with a single analyte. The context of these multiple coding tags depends upon the technique used for signal amplification. Thus, where branched DNA is used, the branched DNA molecule includes the multiple coding tags on the branches. Where oligonucleotide dendrimers are used, the coding tags are on the dendrimer arms. Where rolling circle replication is used, multiple coding tags result from the tandem repeats of complement of the amplification target circle sequence (which includes at least one complement of the coding tag sequence). In this case, the coding tags are tandemly repeated in the tandem sequence DNA.

Oligonucleotide coding tags can each be any length that supports specific and stable hybridization between the coding tags and the decoding tags. For this purpose, a length of 10 to 35 nucleotides is preferred, with a coding tag 15 to 20 nucleotides long being most preferred.

The branched DNA for use in the disclosed method is generally known (Urdea, Biotechnology 12:926-928 (1994), and Horn et al., Nucleic Acids Res 23:4835-4841 (1997)). As used herein, the tail of a branched DNA molecule refers to the portion of a branched DNA molecule that is designed to interact with the analyte. The tail is a

specific binding molecule. In general, each branched DNA molecule should have only one tail. The branches of the branched DNA (also referred to herein as the arms of the branched DNA) contain coding tag sequences. Oligonucleotide dendrimers (or dendrimeric DNA) are also generally known (Shchepinov et al., *Nucleic Acids Res.* 25:4447-4454 (1997), and Örentas et al., *J. Virol. Methods* 77:153-163 (1999)). As used herein, the tail of an oligonucleotide dendrimer refers to the portion of a dendrimer that is designed to interact with the analyte. In general, each dendrimer should have only one tail. The dendrimeric strands of the dendrimer are referred to herein as the arms of the oligonucleotide dendrimer and contain coding tag sequences.

Coding tags can be coupled (directly or via a linker or spacer) to analytes or other molecules to be labeled. Coding tags can also be associated with analytes and other molecules to be labeled. For this purpose, coding molecules are preferred. Coding molecules are molecules that can interact with an analyte and with a decoding tag. Coding molecules include a specific binding molecule and a coding tag. Specific binding molecules are described above.

N. Reporter Carriers and Coding Carriers

Reporter carriers are associations of one or more specific binding molecules, a carrier, and a plurality of reporter signals. Reporter carriers are used in the disclosed method to associate a large number of reporter signals with an analyte. Coding carriers are associations of one or more specific binding molecules, a carrier, and a plurality of coding tags. Coding carriers are used in the disclosed method to associate a large number of coding tags with an analyte. The carrier can be any molecule or structure that facilitates association of many reporter signals with a specific binding molecule. Examples include liposomes, microparticles, nanoparticles, virions, phagmids, and branched polymer structures. A general class of carriers are structures and materials designed for drug delivery. Many such carriers are known. Liposomes are a preferred form of carrier.

Liposomes are artificial structures primarily composed of phospholipid bilayers. Cholesterol and fatty acids may also be included in the bilayer construction. In some forms of the disclosed method, liposomes serve as carriers for arbitrary reporter signals or coding tags. By combining liposome reporter carriers, loaded with arbitrary signals

or tags, with methods capable of separating a very large multiplicity of signals and tags, it becomes possible to perform highly multiplexed assays.

5 Liposomes, preferably unilamellar vesicles, are made using established procedures that result in the loading of the interior compartment with a very large number (several thousand) of reporter signals or coding tag molecules, where the chemical nature of these molecules is well suited for detection by a preselected detection method. One specific type of reporter signal or coding tag preferably is used for each specific type of liposome carrier.

10 Each specific type of liposome reporter or coding carrier is associated with a specific binding molecule. The association may be direct or indirect. An example of a direct association is a liposome containing covalently coupled antibodies on the surface of the phospholipid bilayer. An alternative, indirect association composition is a liposome containing covalently coupled DNA oligonucleotides of arbitrary sequence on its surface; these oligonucleotides are designed to recognize, by base complementarity, 15 specific reporter molecules. The reporter molecule may comprise an antibody-DNA covalent complex, whereby the DNA portion of this complex can hybridize specifically with the complementary sequence on a liposome reporter carrier. In this fashion, the liposome reporter carrier becomes a generic reagent, which may be associated indirectly with any desired binding molecule.

20 The use of liposome reporter carriers can be illustrated with the following example.

1. Liposomes (preferably unilamellar vesicles with an average diameter of 150 to 300 nanometers) are prepared using the extrusion method (Hope et al., *Biochimica et Biophysica Acta*, 812:55-65 (1985); MacDonald et al., *Biochimica et Biophysica* 25 *Acta*, 1061:297-303 (1991)). Other methods for liposome preparation may be used as well.

2. A solution of an oligopeptide, at a concentration 400 micromolar, is used during the preparation of the liposomes, such that the inner volume of the liposomes is loaded with this specific oligopeptide, which will serve to identify a specific analyte of 30 interest. A liposome with an internal diameter of 200 nanometers will contain, on the average, 960 molecules of the oligopeptide. Three separate preparations of liposomes are extruded, each loaded with a different oligopeptide. The oligopeptides are chosen

such that they have the same mass-to-charge ratio but will break into fragments with different mass-to-charge ratios such that they will be readily separable by mass spectrometry.

3. The outer surface of the three liposome preparations is conjugated with specific antibodies, as follows: a) the first liposome preparation is reacted with an antibody specific for the p53 tumor suppressor; b) the second liposome preparation is reacted with an antibody specific for the Bcl-2 oncoprotein; c) the third liposome preparation is reacted with an antibody specific for the Her2/neu membrane receptor. Coupling reactions are performed using standard procedures for the covalent coupling of antibodies to molecules harboring reactive amino groups (Hendrickson et al., *Nucleic Acids Research*, 23:522-529 (1995); Hermanson, *Bioconjugate techniques*, Academic Press, pp.528-569 (1996); Scheffold et al., *Nature Medicine* 1:107-110 (2000)). In the case of the liposomes, the reactive amino groups are those present in the phosphatidyl ethanolamine moieties of the liposomes.

4. A glass slide bearing a standard formaldehyde-fixed histological section is contacted with a mixture of all three liposome preparations, suspended in a buffer containing 30 mM Tris-HCl, pH 7.6, 100 mM Sodium Chloride, 1 mM EDTA, 0.1 % Bovine serum albumin, in order to allow association of the liposomes with the corresponding protein antigens present in the fixed tissue. After a one hour incubation, the slides are washed twice, for 5 minutes, with the same buffer (30 mM Tris-HCl, pH 7.6, 100 mM Sodium Chloride, 1 mM EDTA, 0.1 % Bovine serum albumin). The slides are dried with a stream of air.

5. The slides are coated with a thin layer of matrix solution consisting of 10 mg/ml alpha-cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid in a 50:50 mixture of acetonitrile in water. The slides are dried with a stream of air.

6. The slide is placed on the surface of a MALDI plate, and introduced in a mass spectrometer such as that described in Loboda et al., *Design and Performance of a MALDI-QqTOF Mass Spectrometer*, in *47th ASMS Conference*, Dallas, Texas (1999), Loboda et al., *Rapid Comm. Mass Spectrom.* 14(12):1047-1057 (2000), Shevchenko et al., *Anal. Chem.*, 72: 2132-2142 (2000), and Krutchinsky et al., *J. Am. Soc. Mass Spectrom.*, 11(6):493-504 (2000).

7. Mass spectra are obtained from defined positions on the slide surface. The relative amount of each of the three peaks of reporter signal polypeptides is used to determine the relative ratios of the antigens detected by the liposome-detector complexes.

- 5 The liposome carrier method is not limited to the detection of analytes on histological sections. Cells obtained by sorting may also be used for analysis in the disclosed method (Scheffold, A., Assenmacher, M., Reiners-Schramm, L., Lauster, R., and Radbruch, A., 2000, *Nature Medicine* 1:107-110).

O. Labeled Proteins

- 10 Labeled proteins are proteins or peptides to which one or more reporter signals are attached. Preferably, the reporter signal and the protein or peptide are covalently coupled or tethered to each other. As used herein, molecules are coupled when they are covalent joined, directly or indirectly. One form of indirect coupling is via a linker molecule. The reporter signal can be coupled to the protein or peptide by any suitable
15 coupling reactions. For example, reporter signals can be covalently coupled to proteins through a sulfur-sulfur bond between a cysteine on the protein and a cysteine on the reporter signal. Many other chemistries and techniques for coupling compounds to proteins are known and can be used to couple reporter signals to proteins. For example, coupling can be made using thiols, epoxides, nitriles for thiols, NHS esters,
20 isothiocyanates, isothiocyanates for amines, amines, and alcohols for carboxylic acids. Proteins and peptides can also be labeled in vivo.

- As used herein, "labeled protein" refers to both proteins and peptides to which one or more reporter signals are attached. The term labeled protein refers both to proteins and peptides attached to intact (for example, unfragmented) reporter signals
25 and to proteins and peptides attached to modified (for example, fragmented) reporter signals. The latter form of labeled proteins are referred to as fragmented labeled proteins. Although the protein portion of a labeled protein can be fragmented (for example, by protease digestion), the term fragmented labeled protein refers to a labeled protein where the reporter signal has been fragmented. Isobaric labeled proteins are
30 proteins or peptides of the same type that are labeled with isobaric reporter signals such that a set of the proteins has the same mass-to-charge ratio.

by the viral antigens. Preferred vectors of this type will carry coding regions for Interleukin 8 or 10.

Viral vectors have higher transaction (ability to introduce genes) abilities than do most chemical or physical methods to introduce genes into cells. Typically, viral vectors contain, nonstructural early genes, structural late genes, an RNA polymerase III transcript, inverted terminal repeats necessary for replication and encapsidation, and promoters to control the transcription and replication of the viral genome. When engineered as vectors, viruses typically have one or more of the early genes removed and a gene or gene/promotor cassette is inserted into the viral genome in place of the removed viral DNA. Constructs of this type can carry up to about 8 kb of foreign genetic material. The necessary functions of the removed early genes are typically supplied by cell lines which have been engineered to express the gene products of the early genes in trans.

1. Retroviral Vectors

A retrovirus is an animal virus belonging to the virus family of Retroviridae, including any types, subfamilies, genus, or tropisms. Retroviral vectors, in general, are described by Verma, I.M., Retroviral vectors for gene transfer. In Microbiology-1985, American Society for Microbiology, pp. 229-232, Washington, (1985). Examples of methods for using retroviral vectors for gene therapy are described in U.S. Patent Nos. 4,868,116 and 4,980,286; PCT applications WO 90/02806 and WO 89/07136; and Mulligan, (Science 260:926-932 (1993)).

A retrovirus is essentially a package which has packed into it nucleic acid cargo. The nucleic acid cargo carries with it a packaging signal, which ensures that the replicated daughter molecules will be efficiently packaged within the package coat. In addition to the package signal, there are a number of molecules which are needed in cis, for the replication, and packaging of the replicated virus. Typically a retroviral genome, contains the gag, pol, and env genes which are involved in the making of the protein coat. It is the gag, pol, and env genes which are typically replaced by the foreign DNA that it is to be transferred to the target cell. Retrovirus vectors typically contain a packaging signal for incorporation into the package coat, a sequence which signals the start of the gag transcription unit, elements necessary for reverse transcription, including a primer binding site to bind the tRNA primer of reverse

transcription, terminal repeat sequences that guide the switch of RNA strands during DNA synthesis, a purine rich sequence 5' to the 3' LTR that serve as the priming site for the synthesis of the second strand of DNA synthesis, and specific sequences near the ends of the LTRs that enable the insertion of the DNA state of the retrovirus to insert
5 into the host genome. The removal of the gag, pol, and env genes allows for about 8 kb of foreign sequence to be inserted into the viral genome, become reverse transcribed, and upon replication be packaged into a new retroviral particle. This amount of nucleic acid is sufficient for the delivery of a one to many genes depending on the size of each transcript. It is preferable to include either positive or negative selectable markers
10 along with other genes in the insert.

Since the replication machinery and packaging proteins in most retroviral vectors have been removed (gag, pol, and env), the vectors are typically generated by placing them into a packaging cell line. A packaging cell line is a cell line which has been transfected or transformed with a retrovirus that contains the replication and
15 packaging machinery, but lacks any packaging signal. When the vector carrying the DNA of choice is transfected into these cell lines, the vector containing the gene of interest is replicated and packaged into new retroviral particles, by the machinery provided in cis by the helper cell. The genomes for the machinery are not packaged because they lack the necessary signals.

20 2. Adenoviral Vectors

The construction of replication-defective adenoviruses has been described (Berkner et al., J. Virology 61:1213-1220 (1987); Massie et al., Mol. Cell. Biol. 6:2872-2883 (1986); Haj-Ahmad et al., J. Virology 57:267-274 (1986); Davidson et al., J. Virology 61:1226-1239 (1987); Zhang "Generation and identification of
25 recombinant adenovirus by liposome-mediated transfection and PCR analysis" BioTechniques 15:868-872 (1993)). The benefit of the use of these viruses as vectors is that they are limited in the extent to which they can spread to other cell types, since they can replicate within an initial infected cell, but are unable to form new infectious viral particles. Recombinant adenoviruses have been shown to achieve high efficiency
30 gene transfer after direct, in vivo delivery to airway epithelium, hepatocytes, vascular endothelium, CNS parenchyma and a number of other tissue sites (Morsy, J. Clin. Invest. 92:1580-1586 (1993); Kirshenbaum, J. Clin. Invest. 92:381-387 (1993);

Roessler, J. Clin. Invest. 92:1085-1092 (1993); Moullier, Nature Genetics 4:154-159 (1993); La Salle, Science 259:988-990 (1993); Gomez-Foix, J. Biol. Chem. 267:25129-25134 (1992); Rich, Human Gene Therapy 4:461-476 (1993); Zabner, Nature Genetics 6:75-83 (1994); Guzman, Circulation Research 73:1201-1207 (1993);
5 Bout, Human Gene Therapy 5:3-10 (1994); Zabner, Cell 75:207-216 (1993); Caillaud, Eur. J. Neuroscience 5:1287-1291 (1993); and Ragot, J. Gen. Virology 74:501-507 (1993)). Recombinant adenoviruses achieve gene transduction by binding to specific cell surface receptors, after which the virus is internalized by receptor-mediated endocytosis, in the same manner as wild type or replication-defective adenovirus
10 (Chardonnet and Dales, Virology 40:462-477 (1970); Brown and Burlingham, J. Virology 12:386-396 (1973); Svensson and Persson, J. Virology 55:442-449 (1985); Seth, et al., J. Virol. 51:650-655 (1984); Seth, et al., Mol. Cell. Biol. 4:1528-1533 (1984); Varga et al., J. Virology 65:6061-6070 (1991); Wickham et al., Cell 73:309-319 (1993)).

15 A preferred viral vector is one based on an adenovirus which has had the E1 gene removed and these virions are generated in a cell line such as the human 293 cell line. In another preferred embodiment both the E1 and E3 genes are removed from the adenovirus genome.

Another type of viral vector is based on an adeno-associated virus (AAV). This
20 defective parvovirus is a preferred vector because it can infect many cell types and is nonpathogenic to humans. AAV type vectors can transport about 4 to 5 kb and wild type AAV is known to stably insert into chromosome 19. Vectors which contain this site specific integration property are preferred. An especially preferred embodiment of this type of vector is the P4.1 C vector produced by Avigen, San Francisco, CA, which
25 can contain the herpes simplex virus thymidine kinase gene, HSV-tk, and/or a marker gene, such as the gene encoding the green fluorescent protein, GFP.

The inserted genes in viral and retroviral usually contain promoters, and/or enhancers to help control the expression of the desired gene product. A promoter is generally a sequence or sequences of DNA that function when in a relatively fixed
30 location in regard to the transcription start site. A promoter contains core elements required for basic interaction of RNA polymerase and transcription factors, and may contain upstream elements and response elements.

3. Viral Promoters and Enhancers

Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature*, 273: 113 (1978)). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment (Greenway, P.J. et al., *Gene* 18: 355-360 (1982)). Of course, promoters from the host cell or related species also are useful herein.

Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5' (Laimins, L. et al., *Proc. Natl. Acad. Sci.* 78: 993 (1981)) or 3' (Lusky, M.L., et al., *Mol. Cell Bio.* 3: 1108 (1983)) to the transcription unit. Furthermore, enhancers can be within an intron (Banerji, J.L. et al., *Cell* 33: 729 (1983)) as well as within the coding sequence itself (Osborne, T.F., et al., *Mol. Cell Bio.* 4: 1293 (1984)). They are usually between 10 and 300 bp in length, and they function in cis. Enhancers function to increase transcription from nearby promoters. Enhancers also often contain response elements that mediate the regulation of transcription. Promoters can also contain response elements that mediate the regulation of transcription. Enhancers often determine the regulation of expression of a gene. While many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein and insulin), typically one will use an enhancer from a eukaryotic cell virus. Preferred examples are the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

The promotor and/or enhancer may be specifically activated either by light or specific chemical events which trigger their function. Systems can be regulated by reagents such as tetracycline and dexamethasone. There are also ways to enhance viral vector gene expression by exposure to irradiation, such as gamma irradiation, or alkylating chemotherapy drugs.

It is preferred that the promoter and/or enhancer region act as a constitutive promoter and/or enhancer to maximize expression of the region of the transcription unit to be transcribed. It is further preferred that the promoter and/or enhancer region be active in all eukaryotic cell types. A preferred promoter of this type is the CMV promoter (650 bases). Other preferred promoters are SV40 promoters, cytomegalovirus (full length promoter), and retroviral vector LTF.

It has been shown that all specific regulatory elements can be cloned and used to construct expression vectors that are selectively expressed in specific cell types such as melanoma cells. The glial fibrillary acetic protein (GFAP) promoter has been used to selectively express genes in cells of glial origin.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding tissue factor protein. The 3' untranslated regions also include transcription termination sites. It is preferred that the transcription unit also contain a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. It is preferred that homologous polyadenylation signals be used in the transgene constructs. In a preferred embodiment of the transcription unit, the polyadenylation region is derived from the SV40 early polyadenylation signal and consists of about 400 bases. It is also preferred that the transcribed units contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

4. Markers

The viral vectors can include nucleic acid sequence encoding a marker product. This marker product is used to determine if the gene has been delivered to the cell and once delivered is being expressed. Preferred marker genes are the E. Coli lacZ gene which encodes β -galactosidase and green fluorescent protein. In some embodiments the marker may be a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine

kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are: CHO DHFR⁻ cells and mouse LTK⁻ cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., J. Molec. Appl. Genet. 1: 327 (1982)), mycophenolic acid, (Mulligan, R.C. and Berg, P. Science 209: 1422 (1980)) or hygromycin, (Sugden, B. et al., Mol. Cell. Biol. 5: 410-413 (1985)). The three examples employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puramycin.

Forms and Embodiments of the Disclosed Materials

A. Reporter Molecule Labeling

Disclosed are sets of reporter signals comprising a plurality of reporter signals, wherein the reporter signals have a common property, wherein the common property allows the reporter signals to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signals can be altered, wherein the altered

forms of each reporter signal can be distinguished from every other altered form of reporter signal.

Disclosed are sets of reporter signals, wherein the reporter signals comprise peptides, wherein the peptides have the same mass-to-charge ratio.

5 Also disclosed are sets wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their mass, wherein the altered forms of the reporter signals can be distinguished via differences in the mass-to-charge ratio of the altered forms of reporter signals and sets wherein the mass of the reporter signals is altered by fragmentation.

10 In addition, sets wherein the set comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signals are also disclosed and sets wherein the set comprises ten or more
15 different reporter signals.

Disclosed are sets of wherein the reporter signals are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

Also disclosed are sets wherein the reporter signals are associated with, or coupled to, specific binding molecules, wherein each reporter signal is associated with,
20 or coupled to, a different specific binding molecule.

Also disclosed are sets wherein the reporter signals are associated with, or coupled to, decoding tags, wherein each reporter signal is associated with, or coupled to, a different decoding tag.

Also disclosed are sets wherein the peptides have the same amino acid
25 composition.

Also disclosed are sets wherein the peptides have the same amino acid sequence.

Further disclosed are sets wherein each peptide contains a different distribution of heavy isotopes.

30 Further disclosed are sets wherein each peptide has a different amino acid sequence.

Also disclosed are sets wherein each peptide has a labile or scissile bond in a different location.

Disclosed are kits comprising (a) a set of reporter molecules, wherein each reporter molecule comprises a reporter signal and a decoding tag, wherein the reporter
5 signals have a common property, wherein the common property allows the reporter signals to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signals can be altered, wherein the altered forms of each reporter signal can be distinguished from every other altered form of reporter signal,
10 wherein each different reporter molecule comprises a different decoding tag and a different reporter signal, (b) a set of coding molecules, wherein each coding molecule comprises a specific binding molecule and a coding tag, wherein each specific binding molecule can interact specifically with a different analyte, wherein each coding tag can interact specifically with a different decoding tag.

B. Reporter Signal Protein Labeling

15 Disclosed are sets of labeled proteins wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein the reporter signals have a common property, wherein the common property allows the labeled proteins comprising the same protein or peptide to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signals
20 can be altered, wherein the altered forms of each reporter signal can be distinguished from every other altered form of reporter signal, wherein alteration of the reporter signals alters the labeled proteins, wherein altered forms of each labeled protein can be distinguished from every other altered form of labeled protein.

Disclosed are sets of labeled proteins wherein each labeled protein comprises a
25 protein or peptide and a reporter signal attached to the protein or peptide, wherein the labeled proteins have a common property, wherein the common property allows the labeled proteins comprising the same protein or peptide to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signals can be altered, wherein the altered forms of each reporter signal can be distinguished
30 from every other altered form of reporter signal, wherein alteration of the reporter signals alters the labeled proteins, wherein altered forms of each labeled protein can be distinguished from every other altered form of labeled protein.

Disclosed are sets of labeled proteins wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein the reporter signals can be altered, wherein the altered forms of each reporter signal can be distinguished from every other altered form of reporter signal, wherein alteration of the reporter signals alters the labeled proteins, wherein altered forms of each labeled protein can be distinguished from every other altered form of labeled protein.

Disclosed are sets of labeled proteins wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein the reporter signals have a common property, wherein the common property allows the labeled proteins comprising the same protein or peptide to be distinguished and/or separated from molecules lacking the common property.

Also disclosed are sets wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their mass, wherein the altered forms of the labeled proteins can be distinguished via differences in the mass-to-charge ratio of the altered forms of reporter signals and sets wherein the mass of the reporter signals is altered by fragmentation.

Further disclosed are sets wherein alteration of the reporter signals also alters their charge.

Also disclosed are sets wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their charge, wherein the altered forms of the labeled proteins can be distinguished via differences in the mass-to-charge ratio of the altered forms of reporter signals.

Further disclosed are sets wherein the set comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signals.

Disclosed are sets wherein the set comprises ten or more different reporter signals and sets wherein the reporter signals are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

In addition, disclosed are sets wherein the reporter signals are coupled to the proteins or peptides.

Disclosed are sets wherein the common property allows the labeled proteins to be distinguished and/or separated from molecules lacking the common property.

Also disclosed are sets wherein the common property is one or more affinity tags associated with the reporter signals and sets wherein one or more affinity tags are associated with the reporter signals.

Disclosed are sets of labeled proteins wherein each labeled protein comprises a protein or a peptide and a reporter signal attached to the protein or peptide, wherein the reporter signals comprise peptides, wherein the reporter signal peptides have the same mass-to-charge ratio.

Also disclosed are sets wherein the reporter signal peptides have the same amino acid composition and sets wherein the reporter signal peptides have the same amino acid sequence.

Disclosed are sets wherein each reporter signal peptide contains a different distribution of heavy isotopes and sets wherein each reporter signal peptide contains a different distribution of substituent groups.

Disclosed are sets wherein each reporter signal peptide has a different amino acid sequence and sets wherein each reporter signal peptide has a labile or scissile bond in a different location.

Disclosed are sets wherein one or more affinity tags are associated with the reporter signals.

Disclosed are kits comprising a set of reporter molecules, wherein each reporter molecule comprises a reporter signal and a coupling tag, wherein the reporter signals have a common property, wherein the common property allows the reporter signals to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signals can be altered, wherein the altered forms of each reporter signal can be distinguished from every other altered form of reporter signal, wherein each different reporter molecule comprises a different coupling tag and a different reporter signal.

Disclosed are labeled proteins wherein the labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein the labeled protein has a common property, wherein the common property allows the labeled protein to be distinguished and/or separated from molecules lacking the common

property, wherein the reporter signal can be altered, wherein alteration of the reporter signals alters the labeled protein, wherein altered form of the labeled protein can be distinguished from the unaltered form of labeled protein.

C. Reporter Signal Calibrators

- 5 Disclosed are sets of reporter signal calibrators, wherein each reporter signal calibrator shares a common property with a target protein fragment in a set of target protein fragments, wherein the common property allows the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein
10 fragment and reporter signal calibrator that share a common property correspond to each other, wherein the target protein fragments can be altered, wherein the altered forms of the target protein fragments can be distinguished from the other altered forms of the target protein fragments, wherein the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from
15 the altered form of the target protein fragment with which the reporter signal calibrator shares a common property.

Also disclosed are sets wherein the set includes a predetermined amount of each reporter signal calibrator and sets wherein the amount of at least two of the reporter signal calibrators is different.

- 20 Disclosed are sets wherein the relative amount each reporter signal calibrator is based on the relative amount of each corresponding target protein fragment expected to be in the protein sample.

Also disclosed are sets wherein the amount of each of the reporter signal calibrators is the same.

- 25 Disclosed are sets wherein the target protein fragments and reporter signal calibrators can be altered by fragmentation and sets wherein the target protein fragments and reporter signal calibrators can be altered by cleavage at a photocleavable amino acid.

- 30 Disclosed are sets wherein the target protein fragments and reporter signal calibrators can be fragmented in a collision cell and sets wherein the target protein fragments can be fragmented at an asparagine-proline bond.

Also disclosed are sets wherein the target protein fragments are produced by protease digestion of the protein sample and sets wherein the target protein fragments are produced by digestion of the protein sample with a serine protease.

5 Also disclosed are sets wherein the serine protease is trypsin and sets wherein the target protein fragments are produced by cleavage at a photocleavable amino acid.

Disclosed are sets wherein the common property is mass-to-charge ratio, wherein the target protein fragments and reporter signal calibrators can be altered by altering their mass, their charge, or their mass and charge, wherein the altered forms of the target protein fragments and reporter signal calibrators can be distinguished via
10 differences in the mass-to-charge ratio of the altered forms of the target protein fragments and reporter signal calibrators.

Also disclosed are sets wherein the set of reporter signal calibrators comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more,
15 fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signal calibrators.

Disclosed are sets wherein the set of reporter signal calibrators comprises ten or more different reporter signal calibrators and sets wherein the set of target protein fragments comprises two or more, three or more, four or more, five or more, six or
20 more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different target protein fragments.

Disclosed are sets wherein the reporter signal calibrators comprise peptides, wherein the peptides have the same mass-to-charge ratio as the corresponding target
25 protein fragments.

Also disclosed are sets wherein the peptides have the same amino acid composition as the corresponding target protein fragments.

Also disclosed are sets wherein the peptides have the same amino acid sequence as the corresponding target protein fragments.

30 In addition, sets are disclosed wherein each peptide has a different amino acid sequence than the corresponding target protein fragment.

Furthermore, sets are disclosed wherein each peptide has a labile or scissile bond in a different location.

Disclosed are sets wherein the reporter signal calibrators are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids and sets wherein at least one of the target protein fragments comprises at least one modified amino acid.

Also disclosed are sets wherein the modified amino acid is a phosphorylated amino acid, an acylated amino acid, or a glycosylated amino acid.

Also disclosed are sets wherein at least one of the target protein fragments is the same as the target protein fragment comprising the modified amino acid except for the modified amino acid.

Disclosed are kits for producing a protein signature, the kit comprising (a) a set of reporter signal calibrators, wherein each reporter signal calibrator shares a common property with a target protein fragment in a set of target protein fragments, wherein the common property allows the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein fragment and reporter signal calibrator that share a common property correspond to each other, wherein the target protein fragments can be altered, wherein the altered forms of the target protein fragments can be distinguished from the other altered forms of the target protein fragments, wherein the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the target protein fragment with which the reporter signal calibrator shares a common property, (b) one or more reagents for treating a protein sample to produce protein fragments.

Also disclosed are kits wherein the set of reporter signal calibrators includes a predetermined amount of each reporter signal calibrator.

Also disclosed are kits wherein the amount of at least two of the reporter signal calibrators is different and kits wherein the relative amount each reporter signal calibrator is based on the relative amount of each corresponding target protein fragment expected to be in the protein sample.

Disclosed are kits wherein the amount of each of the reporter signal calibrators is the same and kits wherein the target protein fragments and reporter signal calibrators can be altered by fragmentation.

Also disclosed are kits wherein the target protein fragments and reporter signal
5 calibrators can be altered by cleavage at a photocleavable amino acid and kits wherein the target protein fragments and reporter signal calibrators can be fragmented in a collision cell.

Disclosed are kits wherein the target protein fragments can be fragmented at an asparagine-proline bond and kits wherein the target protein fragments are produced by
10 protease digestion of the protein sample.

Disclosed are kits wherein the target protein fragments are produced by digestion of the protein sample with a serine protease and kits wherein the serine protease is trypsin.

Disclosed are kits wherein the target protein fragments are produced by
15 cleavage at a photocleavable amino acid.

Also disclosed are kits wherein the common property is mass-to-charge ratio, wherein the target protein fragments and reporter signal calibrators can be altered by altering their mass, their charge, or their mass and charge, wherein the altered forms of the target protein fragments and reporter signal calibrators can be distinguished via
20 differences in the mass-to-charge ratio of the altered forms of the target protein fragments and reporter signal calibrators.

Disclosed are kits wherein the set of reporter signal calibrators comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or
25 more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signal calibrators.

Also disclosed are kits wherein the set of reporter signal calibrators comprises ten or more different reporter signal calibrators.

Disclosed are kits wherein the set of target protein fragments comprises two or
30 more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or

more; sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different target protein fragments.

Also disclosed are kits wherein the reporter signal calibrators comprise peptides, wherein the peptides have the same mass-to-charge ratio as the corresponding target protein fragments.

Disclosed are kits wherein the peptides have the same amino acid composition as the corresponding target protein fragments and kits wherein the peptides have the same amino acid sequence as the corresponding target protein fragments and kits wherein each peptide has a different amino acid sequence than the corresponding target protein fragment.

Disclosed are kits wherein each peptide has a labile or scissile bond in a different location.

Also disclosed are kits wherein the reporter signal calibrators are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

Disclosed are kits wherein at least one of the target protein fragments comprises at least one modified amino acid and disclosed are kits wherein the modified amino acid is a phosphorylated amino acid, an acylated amino acid, or a glycosylated amino acid.

Also disclosed are kits wherein at least one of the target protein fragments is the same as the target protein fragment comprising the modified amino acid except for the modified amino acid.

Disclosed are mixtures comprising a set of reporter signal calibrators and a set of target protein fragments, wherein each reporter signal calibrator shares a common property with a target protein fragment in the set of target protein fragments, wherein the common property allows the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein fragment and reporter signal calibrator that share a common property correspond to each other, wherein the target protein fragments can be altered, wherein the altered forms of the target protein fragments can be distinguished from the other altered forms of the target protein fragments, wherein the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the

target protein fragment with which the reporter signal calibrator shares a common property.

Also disclosed are mixtures wherein the set of reporter signal calibrators includes a predetermined amount of each reporter signal calibrator.

5 Also disclosed are mixtures wherein the amount of at least two of the reporter signal calibrators is different and mixtures wherein the relative amount each reporter signal calibrator is based on the relative amount of each corresponding target protein fragment expected to be in the protein sample.

10 Disclosed are mixtures wherein the amount of each of the reporter signal calibrators is the same and mixtures wherein the target protein fragments and reporter signal calibrators can be altered by fragmentation.

Disclosed are mixtures wherein the target protein fragments and reporter signal calibrators can be altered by cleavage at a photocleavable amino acid.

15 Also disclosed are mixtures wherein the target protein fragments and reporter signal calibrators can be fragmented in a collision cell.

Disclosed are mixtures wherein the target protein fragments can be fragmented at an asparagine-proline bond and mixtures wherein the target protein fragments are produced by protease digestion of the protein sample and mixtures wherein the target protein fragments are produced by digestion of the protein sample with a serine protease and mixtures wherein the serine protease is trypsin.

20 Disclosed are mixtures wherein the target protein fragments are produced by cleavage at a photocleavable amino acid and mixtures wherein the common property is mass-to-charge ratio, wherein the target protein fragments and reporter signal calibrators can be altered by altering their mass, their charge, or their mass and charge, wherein the altered forms of the target protein fragments and reporter signal calibrators can be distinguished via differences in the mass-to-charge ratio of the altered forms of the target protein fragments and reporter signal calibrators.

25 Disclosed are mixtures wherein the set of reporter signal calibrators comprises two or more, three or more, four or more, five or more, six or more, seven or more, 30 eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signal calibrators.

Also disclosed are mixtures wherein the set of reporter signal calibrators comprises ten or more different reporter signal calibrators and mixtures wherein the set of target protein fragments comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different target protein fragments.

Disclosed are mixtures wherein the reporter signal calibrators comprise peptides, wherein the peptides have the same mass-to-charge ratio as the corresponding target protein fragments and mixtures wherein the peptides have the same amino acid composition as the corresponding target protein fragments.

Disclosed are mixtures wherein the peptides have the same amino acid sequence as the corresponding target protein fragments and mixtures wherein each peptide has a different amino acid sequence than the corresponding target protein fragment.

Disclosed are mixtures wherein each peptide has a labile or scissile bond in a different location.

Disclosed are mixtures wherein the reporter signal calibrators are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

Also disclosed are mixtures wherein at least one of the target protein fragments comprises at least one modified amino acid and mixtures wherein the modified amino acid is a phosphorylated amino acid, an acylated amino acid, or a glycosylated amino acid and mixtures wherein at least one of the target protein fragments is the same as the target protein fragment comprising the modified amino acid except for the modified amino acid.

Disclosed are sets of target protein fragments, wherein each target protein fragment shares a common property with a reporter signal calibrator in a set of reporter signal calibrators, wherein the common property allows the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein fragment and reporter signal calibrator that share a common property correspond to each other, wherein the target protein fragments can be altered, wherein the altered forms of the target protein fragments can be distinguished from the other altered forms

of the target protein fragments, wherein the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the target protein fragment with which the reporter signal calibrator shares a common property.

5 Also disclosed are sets wherein the set of reporter signal calibrators includes a predetermined amount of each reporter signal calibrator.

Disclosed are sets wherein the amount of at least two of the reporter signal calibrators is different and sets wherein the relative amount each reporter signal calibrator is based on the relative amount of each corresponding target protein fragment
10 expected to be in the protein sample.

Disclosed are sets wherein the amount of each of the reporter signal calibrators is the same and sets wherein the target protein fragments and reporter signal calibrators can be altered by fragmentation.

Disclosed are sets wherein the target protein fragments and reporter signal
15 calibrators can be altered by cleavage at a photocleavable amino acid and sets wherein the target protein fragments and reporter signal calibrators can be fragmented in a collision cell.

Disclosed are sets wherein the target protein fragments can be fragmented at an asparagine-proline bond.

20 Also disclosed are sets wherein the target protein fragments are produced by protease digestion of the protein sample.

Also disclosed are sets wherein the target protein fragments are produced by digestion of the protein sample with a serine protease and sets wherein the serine protease is trypsin and sets wherein the target protein fragments are produced by
25 cleavage at a photocleavable amino acid.

Disclosed are sets wherein the common property is mass-to-charge ratio, wherein the target protein fragments and reporter signal calibrators can be altered by altering their mass, their charge, or their mass and charge, wherein the altered forms of the target protein fragments and reporter signal calibrators can be distinguished via
30 differences in the mass-to-charge ratio of the altered forms of the target protein fragments and reporter signal calibrators.

Also disclosed are sets wherein the set of reporter signal calibrators comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signal calibrators.

Also disclosed are sets wherein the set of reporter signal calibrators comprises ten or more different reporter signal calibrators.

Also disclosed are sets wherein the set of target protein fragments comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different target protein fragments.

Disclosed are sets wherein the reporter signal calibrators comprise peptides, wherein the peptides have the same mass-to-charge ratio as the corresponding target protein fragments.

Also disclosed are sets wherein the peptides have the same amino acid composition as the corresponding target protein fragments.

Disclosed are sets wherein the peptides have the same amino acid sequence as the corresponding target protein fragments and sets wherein each peptide has a different amino acid sequence than the corresponding target protein fragment.

Also disclosed are sets wherein each peptide has a labile or scissile bond in a different location.

Disclosed are sets wherein the reporter signal calibrators are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

Also disclosed are sets wherein at least one of the target protein fragments comprises at least one modified amino acid.

Also disclosed are sets wherein the modified amino acid is a phosphorylated amino acid, an acylated amino acid, or a glycosylated amino acid.

Disclosed are sets wherein at least one of the target protein fragments is the same as the target protein fragment comprising the modified amino acid except for the modified amino acid.

Disclosed are sets of reporter signal calibrators, wherein each reporter signal calibrator shares a common property with a target protein fragment in a set of target protein fragments, wherein the common property allows each of the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein fragment and reporter signal calibrator that share a common property correspond to each other, wherein each of the target protein fragments can be altered, wherein the altered forms of each target protein fragment can be distinguished from every other altered form of target protein fragment, wherein each of the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the target protein fragment with which the reporter signal calibrator shares a common property.

Disclosed are kits for producing a protein signature, the kit comprising (a) a set of reporter signal calibrators, wherein each reporter signal calibrator shares a common property with a target protein fragment in a set of target protein fragments, wherein the common property allows each of the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein fragment and reporter signal calibrator that share a common property correspond to each other, wherein each of the target protein fragments can be altered, wherein the altered forms of each target protein fragment can be distinguished from every other altered form of target protein fragment, wherein each of the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the target protein fragment with which the reporter signal calibrator shares a common property, (b) one or more reagents for treating a protein sample to produce protein fragments.

Disclosed are mixtures comprising a set of reporter signal calibrators and a set of target protein fragments, wherein each reporter signal calibrator shares a common property with a target protein fragment in the set of target protein fragments, wherein the common property allows each of the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein fragment and

reporter signal calibrator that share a common property correspond to each other, wherein each of the target protein fragments can be altered, wherein the altered forms of each target protein fragment can be distinguished from every other altered form of target protein fragment, wherein each of the reporter signal calibrators can be altered, 5 wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the target protein fragment with which the reporter signal calibrator shares a common property.

Also disclosed are sets of target protein fragments, wherein each target protein fragment shares a common property with a reporter signal calibrator in a set of reporter 10 signal calibrators, wherein the common property allows each of the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein fragment and reporter signal calibrator that share a common property correspond to each other, wherein each of the target protein fragments can be altered, 15 wherein the altered forms of each target protein fragment can be distinguished from every other altered form of target protein fragment, wherein each of the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the target protein fragment with which the reporter signal calibrator shares a common property.

20 D. Reporter Signal Fusions

Disclosed are sets of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest, wherein the reporter signal peptides have a common property, wherein the common property allows the reporter signal 25 peptides to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

Also disclosed are sets wherein each nucleic acid molecule further comprises 30 expression sequences, wherein the expression sequences are operably linked to the nucleotide segment such that the amino acid segment can be expressed.

Also disclosed are sets wherein the expression sequences comprise translation expression sequences and sets wherein the expression sequences further comprise transcription expression sequences.

Disclosed are sets wherein the amino acid segment can be expressed in vitro
5 and sets wherein the amino acid segment can be expressed in vivo and sets wherein the amino acid segment can be expressed in cell culture.

Also disclosed are sets wherein the expression sequences of each nucleic acid molecule are different and sets wherein the different expression sequences are differently regulated and sets wherein the expression sequences are similarly regulated
10 and sets wherein a plurality of the expression sequences are expression sequences of, or derived from, genes expressed as part of the same expression cascade and sets wherein the expression sequences of each nucleic acid molecule are the same and sets wherein the expression sequences are similarly regulated and sets wherein the expression sequences of at least two nucleic acid molecules are different and sets wherein the
15 expression sequences of at least two nucleic acid molecules are the same.

Disclosed are sets wherein each nucleic acid molecule further comprises replication sequences, wherein the replication sequences allow replication of the nucleic acid molecules.

Disclosed are sets wherein the nucleic acid molecules can be replicated in vitro
20 and sets wherein the nucleic acid molecules can be replicated in vivo and sets wherein the nucleic acid molecules can be replicated in cell culture.

Disclosed are sets wherein each nucleic acid molecule further comprises integration sequences, wherein the integration sequences allow integration of the nucleic acid molecules into other nucleic acids.

Also disclosed are sets wherein the nucleic acid molecules can be integrated
25 into a chromosome and sets wherein the nucleic acid molecules can be integrated into a chromosome at a predetermined location.

Also disclosed are sets wherein the nucleic acids molecules are produced by replicating nucleic acids in one or more nucleic acid samples.

Also disclosed are sets wherein the nucleic acids are replicated using pairs of
30 primers, wherein each of the first primers in the primer pairs used to produce the nucleic acid molecules comprises a nucleotide sequence encoding the reporter signal

peptide and sets wherein each first primer further comprises expression sequences and sets wherein the nucleotide sequence of each first primer also encodes an epitope tag.

Disclosed are sets wherein each amino acid segment further comprises an epitope tag and sets wherein the epitope tag of each amino acid segment is different and
5 sets wherein the epitope tag of each amino acid segment is the same and sets wherein the epitope tag of at least two amino acid segments are different and sets wherein the epitope tag of at least two amino acid segments are the same.

Disclosed are sets wherein the reporter signal peptide of each amino acid segment is different and sets wherein the reporter signal peptide of each amino acid
10 segment is the same and sets wherein the reporter signal peptide of at least two amino acid segments are different and sets wherein the reporter signal peptide of at least two amino acid segments are the same.

Disclosed are sets wherein the nucleic acid molecules are in cells and sets wherein each nucleic acid molecule is in a different cell and sets wherein each nucleic
15 acid molecule is in the same cell and sets wherein each nucleic acid molecule further comprises expression sequences, wherein the expression sequences are operably linked to the nucleotide segment such that the amino acid segment can be expressed and sets wherein the expression sequences of each nucleic acid molecule are different and sets wherein the expression sequences are similarly regulated and sets wherein a plurality of
20 the expression sequences are expression sequences of, or derived from, genes expressed as part of the same expression cascade.

Disclosed are sets wherein the nucleic acid molecules are integrated into a chromosome of the cell.

Also disclosed are sets wherein the nucleic acid molecules are integrated into
25 the chromosome at a predetermined location.

Disclosed are sets wherein the chromosome is an artificial chromosome.

Disclosed are sets wherein the nucleic acid molecules are, or are integrated into, a plasmid.

Also disclosed are sets wherein the cells are in cell lines.

Also disclosed are sets wherein each nucleic acid molecule is in a different cell
30 line and sets wherein each nucleic acid molecule is in the same cell line.

Disclosed are sets wherein the nucleic acid molecules are in organisms and sets wherein each nucleic acid molecule is in a different organism and sets wherein each nucleic acid molecule is in the same organism and sets wherein each nucleic acid molecule further comprises expression sequences, wherein the expression sequences
5 are operably linked to the nucleotide segment such that the amino acid segment can be expressed and sets wherein the expression sequences of each nucleic acid molecule are different and sets wherein the expression sequences are similarly regulated and sets wherein a plurality of the expression sequences are expression sequences of, or derived from, genes expressed as part of the same expression cascade.

10 Also disclosed are sets wherein the nucleic acid molecules are integrated into a chromosome of the organism.

Disclosed are sets wherein the nucleic acid molecules are integrated into the chromosome at a predetermined location.

Also disclosed are sets wherein the chromosome is an artificial chromosome.

15 Disclosed are sets wherein the nucleic acid molecules are, or are integrated into, a plasmid.

Also disclosed are sets wherein each nucleic acid molecule is in a different organism and sets wherein each nucleic acid molecule is in the same organism and sets wherein the nucleic acid molecules are in cells of an organism and sets wherein the
20 nucleic acid molecules are in substantially all of the cells of the organism and sets wherein the nucleic acid molecules are in some of the cells of the organism.

Disclosed are sets wherein the amino acid segments are expressed in substantially all of the cells of the organism and sets wherein the amino acid segments are expressed in some of the cells of the organism.

25 Disclosed are sets wherein the protein or peptide of interest of each amino acid segment is different and sets wherein the protein or peptide of interest of each amino acid segment is the same and sets wherein the protein or peptide of interest of at least two amino acid segments are different and sets wherein the protein or peptide of interest of at least two amino acid segments are the same and sets wherein the proteins
30 or peptides of interest are related and sets wherein the proteins or peptides of interest are proteins produced in the same cascade and sets wherein the proteins or peptides of interest are proteins expressed under the same conditions and sets wherein the proteins

or peptides of interest are proteins associated with the same disease and sets wherein the proteins or peptides of interest are proteins associated with the same cell type and sets wherein the proteins or peptides of interest are proteins associated with the same tissue type and sets wherein the proteins or peptides of interest are proteins in the same enzymatic pathway.

Disclosed are sets wherein the nucleotide segment encodes a plurality of amino acid segments each comprising a reporter signal peptide and a protein or peptide of interest and sets wherein the protein or peptide of interest of at least two of the amino acid segments in one of the nucleotide segments are different and sets wherein the protein or peptide of interest of the amino acid segments in one of the nucleotide segments are different and sets wherein the protein or peptide of interest of at least two of the amino acid segments in each of the nucleotide segments are different and sets wherein the protein or peptide of interest of the amino acid segments in each of the nucleotide segments are different.

Disclosed are sets wherein the set consists of a single nucleic acid molecule and sets wherein the set consists of a single nucleic acid molecule, wherein the nucleic acid molecule comprises a plurality of nucleotide segments each encoding an amino acid segment and sets wherein the amino acid segment comprises a cleavage site near the junction between the reporter signal peptide and the protein or peptide of interest and sets wherein the cleavage site is a trypsin cleavage site and sets wherein the cleavage site is at the junction between the reporter signal peptide and the protein or peptide of interest.

Disclosed are sets wherein each amino acid segment further comprises a self-cleaving segment.

Disclosed are sets wherein the self-cleaving segment is between the reporter signal peptide and the protein or peptide of interest.

Disclosed are sets wherein the self-cleaving segment is an intein segment.

Disclosed are sets of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest, wherein the reporter signal peptides have a common property, wherein the common property allows the reporter signal peptides to be distinguished and/or separated from molecules lacking the common

property, wherein the reporter signal peptides can be altered, wherein alteration of the reporter signal peptides alters the amino acid segments, wherein the altered form of each amino acid segment can be distinguished from the altered forms of the other amino acid segments.

5 Disclosed are sets of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest, wherein the amino acid segments have a common property, wherein the common property allows the amino acid segments to be distinguished and/or separated from molecules lacking the common
10 property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

 Disclosed are sets of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter
15 signal peptide and a protein or peptide of interest, wherein the amino acid segments have a common property, wherein the common property allows the amino acid segments to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein alteration of the
20 reporter signal peptides alters the amino acid segments, wherein the altered form of each amino acid segment can be distinguished from the altered forms of the other amino acid segments.

 Disclosed are sets of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter
25 signal peptide and a protein or peptide of interest, wherein the amino acid segments each comprise an amino acid subsegment, wherein each amino acid subsegment comprises a portion of the protein or peptide of interest and all or a portion of the reporter signal peptide, wherein the amino acid subsegments have a common property, wherein the common property allows the amino acid subsegments to be distinguished
30 and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

Disclosed are sets of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest, wherein the amino acid segments each comprise an amino acid subsegment, wherein each amino acid subsegment
5 comprises a portion of the protein or peptide of interest and all or a portion of the reporter signal peptide, wherein the amino acid subsegments have a common property, wherein the common property allows the amino acid subsegments to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein alteration of the reporter signal peptides alters
10 the amino acid subsegments, wherein the altered form of each amino acid subsegment can be distinguished from the altered forms of the other amino acid subsegments.

Disclosed are sets of amino acid segments wherein each amino acid segment comprises a reporter signal peptide and a protein or peptide of interest, wherein the reporter signal peptides have a common property, wherein the common property allows
15 the reporter signal peptides to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

Also disclosed are sets wherein the amino acid segment is a protein or peptide
20 and sets wherein the set consists of a single amino acid segment, wherein the amino acid segment comprises a plurality of reporter signal peptides.

Also disclosed cells comprising a set of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest, wherein the
25 reporter signal peptides have a common property, wherein the common property allows the reporter signal peptides to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

30 Disclosed are sets of cells wherein each cell comprises a nucleic acid molecule wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest,

wherein the reporter signal peptides have a common property, wherein the common property allows the reporter signal peptides to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

Also disclosed are sets wherein each cell further comprises additional nucleic acid molecules and sets wherein the set consists of a single cell, wherein the cell comprises a plurality of nucleic acid molecules and sets wherein the set consists of a single cell, wherein the cell comprises a set of nucleic acid molecules, wherein the set of nucleic acid molecules consists of a single nucleic acid molecule, wherein the nucleic acid molecule encodes a plurality of nucleic acid segments.

Disclosed are organisms comprising a set of nucleic acid molecules wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest, wherein the reporter signal peptides have a common property, wherein the common property allows the reporter signal peptides to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

Disclosed are sets of organisms each organism comprises a nucleic acid molecule wherein each nucleic acid molecule comprises a nucleotide segment encoding an amino acid segment comprising a reporter signal peptide and a protein or peptide of interest, wherein the reporter signal peptides have a common property, wherein the common property allows the reporter signal peptides to be distinguished and/or separated from molecules lacking the common property, wherein the reporter signal peptides can be altered, wherein the altered form of each reporter signal peptide can be distinguished from the altered forms of the other reporter signal peptides.

Also disclosed are sets wherein each organism further comprises additional nucleic acid molecules and sets wherein the set consists of a single organism, wherein the organism comprises a plurality of nucleic acid molecules and sets wherein the set consists of a single organism, wherein the organism comprises a set of nucleic acid molecules, wherein the set of nucleic acid molecules consists of a single nucleic acid

molecule, wherein the nucleic acid molecule encodes a plurality of nucleic acid segments.

Method

5 The disclosed methods are useful for sensitive detection of one or multiple analytes. In general, the methods involve the use of special label components, referred to as reporter signals, that can be associated with, incorporated into, or otherwise linked to the analytes, or that can be used merely in conjunction with analytes, with no significant association between the analytes and reporter signals. In some embodiments
10 of the methods, the reporter signals (or derivatives of the reporter signals) are detected, thus indicating the presence of the associated analytes. In other embodiments, the analyte (or derivatives of the analytes) are detected along with the reporter signals (or derivatives of the reporter signals).

 In some embodiments of the methods, the reporter signals can be altered such
15 that the altered forms of different reporter signals can be distinguished from each other. Reporter signal/analyte conjugates can be altered, generally through alteration of the reporter signal portion of the conjugate, such that the altered forms of different reporter signals, altered forms of different reporter signal/analyte conjugates, or both, can be distinguished from each other. Where the reporter signal or reporter signal/analyte
20 conjugate is altered by fragmentation, any, some, or all of the fragments can be distinguished from each other, depending on the embodiment. For example, where reporter signals fragmented into two parts, either or both parts of the reporter signals can be distinguished. Where reporter signal/analyte conjugates are fragmented into two parts (with the break point in the reporter signal portion), either the reporter signal
25 fragment, the reporter signal/analyte fragment, or both can be distinguished. In some embodiments, only one part of a fragmented reporter signal will be detected and so only this part of the reported signals need be distinguished.

 In some embodiments of the methods, sets of reporter signals can be used where
30 two or more of the reporter signals in a set have one or more common properties that allow the reporter signals having the common property to be distinguished and/or separated from other molecules lacking the common property. In other embodiments, sets of reporter signal/analyte conjugates can be used where two or more of the reporter

signal/analyte conjugates in a set have one or more common properties that allow the reporter signal/analyte conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property. In still other embodiments, analytes can be fragmented (prior to or following conjugation) to produce reporter signal/analyte fragment conjugates (which can be referred to as fragment conjugates). In such cases, sets of fragment conjugates can be used where two or more of the fragment conjugates in a set have one or more common properties that allow the fragment conjugates having the common property to be distinguished and/or separated from other molecules lacking the common property.

As indicated above, reporter signals conjugated with analytes can be altered while in the conjugate and distinguished. Conjugated reporter signals can also be dissociated or separated, in whole or in part, from the conjugated analytes prior to their alteration. Where the reporter signals are dissociated (in whole or in part) from the analytes, the method can be performed such that the fact of association between the analyte and reporter signal is part of the information obtained when the reporter signal is detected. In other words, the fact that the reporter signal may be dissociated from the analyte for detection does not obscure the information that the detected reporter signal was associated with the analyte.

Reporter signals can also be in conjunction with analytes (such as in mixtures of reporter signals and analytes), where no significant physical association between the reporter signals and analytes occurs; or alone, where no analyte is present. In such cases, where reporter signals are not or are no longer associated with analytes, sets of reporter signals can be used where two or more of the reporter signals in a set have one or more common properties that allow the reporter signals having the common property to be distinguished and/or separated from other molecules lacking the common property.

In preferred embodiments, the disclosed methods involves two basic steps. A filtering, selection, or separation step to separate reporter signals from other molecules that may be present, and a detection step that distinguishes different reporter signals. The reporter signals preferably are distinguished and/or separated from other molecules based on some common property shared by the reporter signals but not present in most (or, preferably, all) other molecules present. The separated reporter signals are then

treated and/or detected such that the different reporter signals are distinguishable. Useful forms of the disclosed method involve association of reporter signals with analytes of interest. Detection of the reporter signals results in detection of the corresponding analytes. Thus, the disclosed method is a general technique for labeling and detection of analytes.

A preferred form of the disclosed method involves filtering of isobaric reporter signals from other molecules based on mass-to-charge ratio, fragmentation of the reporter signals to produce fragments having different masses, and detection of the different fragments based on their mass-to-charge ratios. The method is best carried out using a tandem mass spectrometer. There are two types of tandem mass spectrometers, as well as hybrids and combinations of these types: "tandem in space" spectrometers and "tandem in time" spectrometers. Tandem in space spectrometers utilize spatially ordered elements and act upon the ions in turn as the ions pass through each element. Tandem in time spectrometers utilize temporally ordered manipulations on the ions as the ions are contained in a space. In a tandem in space class of instrument, the isobaric reporter signals are first passed through a filtering quadrupole, the reporter signals are fragmented (preferably in a collision cell), and the fragments are distinguished and detected in a time-of-flight (TOF) stage. In such an instrument the sample is ionized in the source (for example, in a MALDI) to produce charged ions. It is preferred that the ionization conditions are such that primarily a singly charged parent ion is produced. A first quadrupole, Q0, is operated in radio frequency (RF) mode only and acts as an ion guide for all charged particles. The second quadrupole, Q1, is operated in RF + DC mode to pass only a narrow range of mass-to-charge ratios (that includes the mass-to-charge ratio of the reporter signals). This quadrupole selects the mass-to-charge ratio of interest. Quadrupole Q2, surrounded by a collision cell, is operated in RF only mode and acts as ion guide. The collision cell surrounding Q2 can be filled to appropriate pressure with a gas to fracture the input ions by collisionally induced dissociation. The collision gas preferably is chemically inert, but reactive gases can also be used. Preferred molecular systems utilize reporter signals that contain scissile bonds, labile bonds, or combinations, such that these bonds will be preferentially fractured in the Q2 collision cell.

The same sample can be analyzed both with and without fragmentation (by operating with and without collision gas), and the results compared to detect shifts in mass-to-charge ratio. Both the unfragmented and fragmented results should give diagnostic peaks, with the combination of peaks both with and without fragmentation confirming the reporter signal (and analyte) involved. Such distinctions are accomplished by using appropriate sets of isobaric reporter signals and allows large scale multiplexing in the detection of analytes.

The disclosed method is particularly well suited to the use of a MALDI-QqTOF mass spectrometer. The method enables highly multiplexed analyte detection, and very high sensitivity. Preferred tandem mass spectrometers are described by Loboda *et al.*, *Design and Performance of a MALDI-QqTOF Mass Spectrometer*, in 47th ASMS Conference, Dallas, Texas (1999), Loboda *et al.*, *Rapid Comm. Mass Spectrom.* 14(12):1047-1057 (2000), Shevchenko *et al.*, *Anal. Chem.*, 72: 2132-2142 (2000), and Krutchinsky *et al.*, *J. Am. Soc. Mass Spectrom.*, 11(6):493-504 (2000). In such an instrument the sample is ionized in the source (MALDI, for example) to produce charged ions; it is preferred that the ionization conditions are such that primarily a singly charged parent ion is produced. First and third quadrupoles, Q0 and Q2, will be operated in RF only mode and will act as ion guides for all charged particles, second quadrupole Q1 will be operated in RF + DC mode to pass only a particular mass-to-charge (or, in practice, a narrow mass-to-charge range). This quadrupole selects the mass-to-charge ratio, (m/z), of interest. The collision cell surrounding Q2 can be filled to appropriate pressure with a gas to fracture the input ions by collisionally induced dissociation (normally the collision gas is chemically inert, but reactive gases are contemplated). Preferred molecular systems utilize reporter signals that contain scissile bonds, labile bonds, or combinations, and these bonds will be preferentially fractured in the Q2 collision cell.

A MALDI source is preferred for the disclosed method because it facilitates the multiplexed analysis of samples from heterogeneous environments such as arrays, beads, microfabricated devices, tissue samples, and the like. An example of such an instrument is described by Qin *et al.*, *A practical ion trap mass spectrometer for the analysis of peptides by matrix-assisted laser desorption/ionization.*, *Anal. Chem.*, 68:1784 – 1791 (1996). For homogeneous assays electrospray ionization (ESI) sources

will work very well. Electrospray ionization source instruments interfaced to LC systems are commercially available (for example, QSTAR from PE-SCIEX, Q-TOF from Micromass). It is of note that the ESI sources are operated such that they tend to produce multiply charged ions, doubly charged ions would be most common for ions in the disclosed method. Such doubly charged ions are well known in the art and present no limitation to the disclosed method. TOF analyzers and quadrupole analyzers are preferred detectors over sector analyzers. Tandem in time ion trap systems such as Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometers also may be used with the disclosed method.

A number of elements contribute to the sensitivity of the disclosed method. The filter quadrupole, Q1, selects a narrow mass-to-charge ratio and discriminates against other mass-to-charge ions, significantly decreasing background from non germane ions. For example, for a sample containing a distribution of mass-to-charges of width 3000 Da, a mass-to-charge transmission window of 2 Da applied to this distribution can improve the signal to noise by at least a factor of $3000/2 = 1500$. Once the parent ion is selected by quadrupole Q1, fragmentation of the parent ion, preferably into a single charged daughter ion, has the advantage over systems which fragment the parent into a number of daughter ions. For example, a parent fragmented into 20 daughter ions will yield signals that are on average $1/20^{\text{th}}$ the intensity of the parent ions. For a parent to single daughter system there will not be this signal dilution.

This preferred system for use with the disclosed method has a high duty cycle, and as such good statistics can be collected quickly. For the case where a single set of isobaric parents is used, the multiplexed detection is accomplished without having to scan the filter quadrupole (although such a scan is useful for single pass analysis of a complex protein sample with multiple labeled proteins). Electrospray sources can operate continuously, MALDI sources can operate at several kHz, quadrupoles operate continuously, and time of flight analyzers can capture the entire mass-to-charge region of interest at several kHz repetition rate. Thus, the overall system can acquire thousands of measurements per second. For throughput advantage in a multiplexed assay the time of flight analyzer has an advantage over a quadrupole analyzer for the final stage because the time of flight analyzer detects all fragment ions in the same

acquisition rather than requiring scanning (or stepping) over the ions with a quadrupole analyzer.

Instrumental improvements including addition of laser ports along the flight path to allow intersection of the proteins with additional laser(s) open additional
5 fragmentation avenues through photochemical and photophysical processes (for example, selective bond cleavage, selective ionization). Use of lasers to fragment the proteins after the filter stage will enable the use of the very high throughput TOF-TOF instruments (50 kHz to 100 kHz systems).

The disclosed method is compatible with techniques involving cleavage,
10 treatment, or fragmentation of a bulk sample in order to simplify the sample prior to introduction into the first stage of a multistage detection system. The disclosed method is also compatible with any desired sample, including raw extracts and fractionated samples.

A. Reporter Molecule Labeling

15 In one form of the disclosed method, referred to as reporter molecule labeling, reporter signals are associated with analytes to be detected and/or quantitated. For example, a reporter signal can be associated with a specific binding molecule that interacts with the analyte of interest. Such a combination is referred to as a reporter molecule. The specific binding molecule in the reporter molecule interacts with the
20 analyte thus associating the reporter signal with the analyte. Alternatively, a reporter signal can be associated with an analyte indirectly. In this mode, a "coding" molecule containing a specific binding molecule and a coding tag is associated with the analyte (via the specific binding molecule). Alternatively, a coding tag can be coupled or directly associated with the analyte. Then a reporter signal associated with a decoding
25 tag (such a combination is another form of reporter molecule) is associated with the coding molecule through an interaction between the coding tag and the decoding tag. An example of this interaction is hybridization where the coding and decoding tags are complementary nucleic acid sequences. The result is an indirect association of the reporter signal with the analyte. This mode has the advantage that all of the
30 interactions of the reporter signals with the coding molecule can be made chemically and physically similar by using the same types of coding tags and decoding tags for all of the coding molecules and reporter molecules in a set.

The disclosed method increases the sensitivity and accuracy of detection of an analyte of interest. Preferred forms of the disclosed method make use of multistage detection systems to increase the resolution of the detection of molecules having very similar properties. The method involves at least two stages. The first stage is filtration or selection that allows passage or selection of reporter signals (that is, a subset of the molecules present), based upon intrinsic properties of the reporter signals, and discrimination against all other molecules. The subsequent stage(s) further separate(s) and/or detect(s) the reporter signals which were filtered in the first stage. A key facet of this method is that a multiplexed set of reporter signals will be selected by the filter and subsequently cleaved, decomposed, reacted, or otherwise modified to realize the identities and/or quantities of the reporter signals in further stages. There is a correspondence between the specific binding molecule and the detected daughter fragment.

B. Reporter Signal Labeling

Another form of the disclosed method, referred to as reporter signal labeling, involves detection of analytes by detecting a reporter signal, labeled analyte, or both; or by distinguishing different reporter signals, different labeled analytes, or both. Detection of the reporter signals results in detection of the corresponding labeled analytes (where the analytes are labeled with the reporter signals). Detection of the labeled analytes results in detection of the corresponding analytes. Thus, reporter signal labeling is a general technique for labeling, detection, and quantitation of analytes.

In one embodiment, the disclosed method can involve two basic steps. A filtering, selection, or separation step to separate labeled analytes from other molecules that may be present, and a detection step that detects a reporter signal, labeled analyte, or both; or that distinguishes different reporter signals, different labeled analytes, or both. The labeled analytes preferably are distinguished and/or separated from other molecules based on some common property shared by the attached reporter signals but not present in most (or, preferably, all) other molecules present. The labeled analytes can also be distinguished and/or separated from other molecules based on a common property of the labeled analyte as a whole, such as the mass-to-charge ratio of the labeled analyte. The separated labeled analytes are then treated and/or detected in such

a way that the different reporter signals, different labeled analytes, or both, are distinguishable.

Reporter signals can be coupled or directly associated with an analyte. For example, a reporter signal can be coupled to an analyte via reactive groups, or a reporter molecule (composed of a specific binding molecule and a reporter signal) can be associated with an analyte. Alternatively, a reporter signal can be associated with an analyte indirectly. In this mode, a "coding" molecule containing a specific binding molecule and a coding tag can be associated with the analyte (via the specific binding molecule). Alternatively, a coding tag can be coupled or directly associated with the analyte. Then a reporter signal associated with a decoding tag (such a combination is another form of reporter molecule) is associated with the coding molecule through an interaction between the coding tag and the decoding tag. An example of this interaction is hybridization where the coding and decoding tags are complementary nucleic acid sequences. The result is an indirect association of the reporter signal with the analyte. This mode has the advantage that all of the interactions of the reporter signals with the coding molecule can be made chemically and physically similar by using the same types of coding tags and decoding tags for all of the coding molecules and reporter molecules in a set.

Reporter signals, or constructs containing reporters signals, also can be attached or coupled to analytes by ligation. Methods for ligation of nucleic acids are well known (see, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, second edition, 1989, Cold Spring Harbor Laboratory Press, New York.), and efficient protein ligation is known (see, for example, Dawson et al., "Synthesis of proteins by native chemical ligation" *Science* 266, 776-9 (1994); Hackeng et al., "Chemical synthesis and spontaneous folding of a multidomain protein: anticoagulant microprotein S" *Proc Natl Acad Sci USA* 97:14074-8 (2000); Dawson et al., "Synthesis of Native Proteins by Chemical Ligation" *Ann. Rev. Biochem.* 69:923-960 (2000); U.S. Patent No. 6,184,344; PCT Publication WO 98/28434).

The disclosed method can be used in many modes. For example, the disclosed method can be used to detect a specific analyte (in a specific sample or in multiple samples) or multiple analytes (in a single sample or multiple samples). In each case, the analyte(s) to be detected can be separated either from other, unlabeled analytes or

from other molecules lacking a property of the labeled analyte(s) to be detected. For example, analytes in a sample can be generally labeled with reporter signals and some analytes can be separated on the basis of some property of the analytes. For example, the separated analytes could have a certain mass-to-charge ratio (separation based on mass-to-charge ratio will select both labeled and unlabeled analytes having the selected mass-to-charge ratio). As another example, all of the labeled analytes can be distinguished and/or separated from unlabeled molecules based on a feature of the reporter signal such as an affinity tag. Where different affinity tags are used, some labeled analytes can be distinguished and/or separated from others.

In one mode of the disclosed method, multiple analytes in multiple samples are labeled where all of the analytes in a given sample are labeled with the same reporter signal. That is, the reporter signal is used as a general label of the analytes in a sample. Each sample, however, uses a different reporter signal. This allows samples as a whole to be compared with each other. By additionally separating or distinguishing different analytes in the samples, one can easily analyze many analytes in many samples in a single assay. For example, proteins in multiple samples can be labeled with reporter signals as described above, and the samples mixed together. If some or all of the various labeled proteins are separated by, for example, association of the proteins with antibodies on an array, the presence and amount of a given protein in each of the samples can be determined by identifying the reporter signals present at each array element. If the protein corresponding to a given array element was present in a particular sample, then some of the protein associated with that array element will be labeled with the reporter signal used to label that particular sample. Detection of that reporter signal will indicate this. This same relationship holds true for all of the other samples. Further, the amount of reporter signal detected can indicate the amount of a given protein in a given sample, and the simultaneous quantitation of protein in multiple samples can provide a particularly accurate comparison of the levels of the proteins in the various samples.

Optionally, the selection step can be preceded by fractionation step where a subset of analytes, including the analytes that are, or will be, labeled, are separated from other components in a sample. Such a step, although not necessary, can improve the selection step by reducing the number of extraneous molecules present.

A preferred form of the disclosed method involves filtering of isobaric labeled analytes from other molecules based on mass-to-charge ratio, fragmentation of the reporter signals to produce fragments having different mass-to-charge ratios, and detection of the different fragments based on their mass-to-charge ratios. The different fragments will include the fragment of the reporter signal and the fragmented labeled analyte (made up of the analyte and the remaining part of the reporter signal). Either or both may be detected and will be characteristic of the initial labeled analyte. The method is best carried out using a tandem mass spectrometer. In such an instrument the isobaric reporter signals are first filtered, then reporter signals are fragmented (preferably by collision), and the fragments are distinguished and detected.

A preferred form of the disclosed method involves detection of labeled analytes in two or more samples in the same assay. This allows simple and consistent detection of differences between the analytes in the samples. Differential detection is accomplished by labeling the analytes in each sample with a different reporter signal. Preferably, the different reporter signals used for the different samples will make up an isobaric set. In this way, the same labeled analyte in each sample will have the same mass-to-charge ratio as that labeled analyte in a different sample. Upon fragmentation of the reporter signals, however, each of the fragmented labeled analytes in the different samples will have a different mass-to-charge ratio and thus each can be separately detected. All can be detected in the same measurement. This is a tremendous advantage in both time and quality of the data. For example, since the samples are assayed in a single run, there is no need to correct or normalize the results of different samples assayed in different runs. This allows accurate comparisons of the relative amounts of the same analyte in different samples since that are measured in the same run. There would be no differences to cause inconsistency between the samples.

A preferred use for this multiple sample mode of the disclosed method is the analysis of a time series of samples. Such series are useful for detecting changes in a sample or reaction over time. For example, changes in analyte levels in a cell culture over time after addition of a test compound can be assessed. In this mode, different time point samples are labeled with different reporter signals, preferably making up an isobaric set. In this way, the same labeled analyte for each time point will have the same mass-to-charge ratio as that labeled analyte from a different time point. Upon

fragmentation of the reporter signals, however, each of the fragmented labeled analytes from the different time points will have a different mass-to-charge ratio and thus each can be separately detected.

5 The disclosed method can also be used to gather and catalog information about unknown analytes. This analyte discovery mode can easily link the presence or pattern of analytes with their analysis. For example, a sample of labeled analytes can be compared to analytes in one or more other samples. Analytes that appear in one or some samples but not others can be analyzed using conventional techniques. The object analytes will be distinguishable from others by virtue of the disclosed labeling, 10 detection, and quantitation. This mode of the method is preferably carried out using mass spectrometry.

The disclosed method increases the sensitivity and accuracy of detection of analytes of interest. Preferred forms of the disclosed method make use of multistage detection systems to increase the resolution of the detection of molecules having very 15 similar properties. The method can involve at least two stages. The first stage is filtration or selection that allows passage or selection of labeled analytes (that is, a subset of the molecules present), based upon intrinsic properties of the reporter signals (and the attached analytes), and discrimination against all other molecules. The subsequent stage(s) further separate(s) and/or detect(s) the labeled analytes that were 20 filtered in the first stage. A key facet of this method is that a multiplexed set of labeled analytes will be selected by the filter and the attached reporter signals subsequently will be cleaved, decomposed, reacted, or otherwise modified to realize the identities and/or quantities of the fragmented reporter signals and/or the fragmented labeled analytes in further stages. There is a correspondence between the reporter signal and the detected 25 daughter fragment(s).

In some embodiments, the disclosed method allows a complex sample of analytes to be quickly and easily cataloged in a reproducible manner. Such a catalog can be compared with other, similarly prepared catalogs of other analyte samples to allow convenient detection of differences between the samples. The catalogs, which 30 incorporate a significant amount of information about the analyte samples, can serve as fingerprints of the samples which can be used both for detection of related analyte samples and comparison of analyte samples. For example, the presence or identity of

specific organisms can be detected by producing a catalog of analytes of the test organism and comparing the resulting catalog with reference catalogs prepared from known organisms. Changes and differences in analyte patterns can also be detected by preparing catalogs of analytes from different cell samples and comparing the catalogs.

- 5 Comparison of analyte catalogs produced with the disclosed method is facilitated by the fine resolution that can be provided with, for example, mass spectrometry.

Each labeled analyte processed in the disclosed method will result in a signal based on the characteristics of the labeled analyte (for example, the mass-to-charge ratio). A complex analyte sample can produce a unique pattern of signals. It is this
10 pattern that can allow unique cataloging of analyte samples and sensitive and powerful comparisons of the patterns of signals produced from different analyte samples.

The presence, amount, presence and amount, or absence of different labeled analytes forms a pattern of signals that provides a signature or fingerprint of the analytes, and thus of the analyte sample based on the presence or absence of specific
15 analytes or analyte fragments in the sample. For this reason, cataloging of this pattern of signals (that is, the pattern of the presence, amount, presence and amount, or absence of labeled analytes) is an embodiment of the disclosed method that is of particular interest.

Catalogs can be made up of, or be referred to, as, for example, a pattern of
20 labeled analytes, a pattern of the presence of labeled analytes, a catalog of labeled analytes, or a catalog of analytes in a sample. The information in the catalog is preferably in the form of mass-to-charge information or compositional information. Catalogs can also contain or be made up of other information derived from the information generated in the disclosed method (for example, the identity of the analytes
25 detected), and can be combined with information obtained or generated from any other source. The informational nature of catalogs produced using the disclosed method lends itself to combination and/or analysis using known bioinformatics systems and methods.

Such catalogs of analyte samples can be compared to a similar catalog derived
30 from any other sample to detect similarities and differences in the samples (which is indicative of similarities and differences in the analytes in the samples). For example, a catalog of a first analyte sample can be compared to a catalog of a sample from the

same type of organism as the first analyte sample, a sample from the same type of tissue as the first analyte sample, a sample from the same organism as the first analyte sample, a sample obtained from the same source but at time different from that of the first analyte sample, a sample from an organism different from that of the first analyte sample, a sample from a type of tissue different from that of the first analyte sample, a sample from a strain of organism different from that of the first analyte sample, a sample from a species of organism different from that of the first analyte sample, or a sample from a type of organism different from that of the first analyte sample.

The same type of tissue is tissue of the same type such as liver tissue, muscle tissue, or skin (which may be from the same or a different organism or type of organism). The same organism refers to the same individual, animal, or cell. For example, two samples taken from a patient are from the same organism. The same source is similar but broader, referring to samples from, for example, the same organism, the same tissue from the same organism, the same analyte, or the same analyte sample. Samples from the same source that are to be compared can be collected at different times (thus allowing for potential changes over time to be detected). This is especially useful when the effect of a treatment or change in condition is to be assessed. Samples from the same source that have undergone different treatments can also be collected and compared using the disclosed method. A different organism refers to a different individual organism, such as a different patient, a different individual animal. Different organism includes a different organism of the same type or organisms of different types. A different type of organism refers to organisms of different types such as a dog and cat, a human and a mouse, or *E. coli* and *Salmonella*. A different type of tissue refers to tissues of different types such as liver and kidney, or skin and brain. A different strain or species of organism refers to organisms differing in their species or strain designation as those terms are understood in the art.

When comparing catalogs of analytes obtained from related samples, it is possible to identify the presence of a subset of correlated pairs of labeled analytes and their altered forms. The disclosed method can be used to detect the original labeled analytes (and determine characteristics of them) and the altered form of the labeled

analytes. This pattern of detected analytes will be characteristic of the analyte that is labeled and the specific reporter signal used (although not necessarily unique).

1. Reporter Signal Protein Labeling

One form of reporter signal labeling, referred to as reporter signal protein
5 labeling, involves detection of proteins by detecting a reporter signal, labeled protein, or both; or by distinguishing different reporter signals, different labeled proteins, or both. Detection of the reporter signals results in detection of the corresponding labeled proteins. Detection of the labeled proteins results in detection of the corresponding
10 proteins. Thus, the disclosed method is a general technique for labeling, detection, and quantitation of proteins.

In one embodiment, the disclosed method can involve two basic steps. A filtering, selection, or separation step to separate labeled proteins from other molecules that may be present, and a detection step that detects a reporter signal, labeled protein, or both; or that distinguishes different reporter signals, different labeled proteins, or
15 both. The labeled proteins preferably are distinguished and/or separated from other molecules based on some common property shared by the attached reporter signals but not present in most (or, preferably, all) other molecules present. The labeled proteins can also be distinguished and/or separated from other molecules based on a common property of the labeled protein as a whole, such as the mass-to-charge ratio of the
20 labeled protein. The separated labeled proteins are then treated and/or detected in such a way that the different reporter signals, different labeled proteins, or both, are distinguishable.

The disclosed method can be used in many modes. For example, the disclosed method can be used to detect a specific protein (in a specific sample or in multiple
25 samples) or multiple proteins (in a single sample or multiple samples). In each case, the protein(s) to be detected can be separated either from other, unlabeled proteins or from other molecules lacking a property of the labeled protein(s) to be detected. For example, proteins in a sample can be generally labeled with reporter signals and some proteins can be separated on the basis of some property of the proteins. For example,
30 the separated proteins could have a certain mass-to-charge ratio (separation based on mass-to-charge ratio will select both labeled and unlabeled proteins having the selected mass-to-charge ratio). As another example, all of the labeled proteins can be

distinguished and/or separated from unlabeled molecules based on a feature of the reporter signal such as an affinity tag. Where different affinity tags are used, some labeled proteins can be distinguished and/or separated from others.

5 Optionally, the selection step can be preceded by fractionation step where a subset of proteins, including the proteins that are, or will be, labeled, are separated from other components in a sample. For example, proteins having an SH2 domain can be separated from other proteins in a cell sample prior to the selection step. Such a step, although not necessary, can improve the selection step by reducing the number of extraneous molecules present.

10 A preferred form of the disclosed method involves filtering of isobaric labeled proteins from other molecules based on mass-to-charge ratio, fragmentation of the reporter signals to produce fragments having different mass-to-charge ratios, and detection of the different fragments based on their mass-to-charge ratios. The different fragments will include the fragment of the reporter signal and the fragmented labeled
15 protein (made up of the protein and the remaining part of the reporter signal). Either or both may be detected and will be characteristic of the initial labeled protein. The method is best carried out using a tandem mass spectrometer. In such an instrument the isobaric reporter signals are first filtered, then reporter signals are fragmented (preferably by collision), and the fragments are distinguished and detected.

20 The same sample can be analyzed both with and without fragmentation (by operating with and without collision gas), and the results compared to detect shifts in mass-to-charge ratio. Both the unfragmented and fragmented results should give diagnostic peaks, with the combination of peaks both with and without fragmentation confirming the identity of the protein (and reporter signal) involved. Such distinctions
25 are accomplished by using appropriate sets of isobaric reporter signals and allows large scale multiplexing in the detection of proteins.

30 A preferred form of the disclosed method involves detection of labeled proteins in two or more samples in the same assay. This allows simple and consistent detection of differences between the proteins in the samples. Differential detection is accomplished by labeling the proteins in each sample with a different reporter signal. Preferably, the different reporter signals used for the different samples will make up an isobaric set. In this way, the same labeled protein in each sample will have the same

mass-to-charge ratio as that labeled protein in a different sample. Upon fragmentation of the reporter signals, however, each of the fragmented labeled proteins in the different samples will have a different mass-to-charge ratio and thus each can be separately detected. All can be detected in the same measurement. This is a tremendous
5 advantage in both time and quality of the data. For example, since the samples are assayed in a single run, there is no need to correct or normalize the results of different samples assayed in different runs. This allows accurate comparisons of the relative amounts of the same protein or peptide in different samples since that are measured in the same run. There would be no differences to cause inconsistency between the
10 samples.

A preferred use for this multiple sample mode of the disclosed method is the analysis of a time series of samples. Such series are useful for detecting changes in a sample or reaction over time. For example, changes in expressed proteins in a cell culture over time after addition of a test compound can be assessed. In this mode,
15 different time point samples are labeled with different reporter signals, preferably making up an isobaric set. In this way, the same labeled protein for each time point will have the same mass-to-charge ratio as that labeled protein from a different time point. Upon fragmentation of the reporter signals, however, each of the fragmented labeled proteins from the different time points will have a different mass-to-charge ratio
20 and thus each can be separately detected.

The disclosed method can also be used to gather and catalog information about unknown proteins. This protein discovery mode can easily link the presence or pattern of proteins with their analysis. For example, a sample of labeled proteins can be compared to proteins in one or more other samples. Proteins that appear in one or some
25 samples but not others can be analyzed for composition and/or sequence using conventional techniques. The object proteins will be distinguishable from others by virtue of the disclosed labeling, detection, and quantitation. This mode of the disclosed method is especially useful as an aid to functional genomics or proteomics since proteins discovered to differ between samples can be characterized. This mode of the
30 method is preferably carried out using mass spectrometry.

The disclosed method increases the sensitivity and accuracy of detection of proteins of interest. Preferred forms of the disclosed method make use of multistage

P. Labeled Analytes

Labeled analytes are analytes to which one or more reporter signals are attached. Preferably, the reporter signal and the analyte are covalently coupled or tethered to each other. As used herein, molecules are coupled when they are covalent
5 joined, directly or indirectly. One form of indirect coupling is via a linker molecule. The reporter signal can be coupled to the analyte by any suitable coupling reactions. Many chemistries and techniques for coupling compounds are known and can be used to couple reporter signals to analytes. For example, coupling can be made using thiols, epoxides, nitriles for thiols, NHS esters, isothiocyanates, isothiocyanates for amines,
10 amines, and alcohols for carboxylic acids.

As used herein, "labeled analyte" refers to analytes to which one or more reporter signals are attached. The term labeled analyte refers both to analytes attached to intact (for example, unfragmented) reporter signals and to analytes attached to modified (for example, fragmented) reporter signals. The latter form of labeled
15 proteins are referred to as fragmented labeled analytes. Although the analyte portion of a labeled analyte can be fragmented, the term fragmented labeled analyte refers to a labeled analyte where the reporter signal has been fragmented. Isobaric labeled analytes are analytes of the same type that are labeled with isobaric reporter signals such that a set of the analytes has the same mass-to-charge ratio.

20 Q. Affinity Tags

An affinity tag is any compound that can be used to separate compounds or complexes having the affinity tag from those that do not. Preferably, an affinity tag is a compound, such as a ligand or hapten, that associates or interacts with another compound, such as ligand-binding molecule or an antibody. It is also preferred that
25 such interaction between the affinity tag and the capturing component be a specific interaction, such as between a hapten and an antibody or a ligand and a ligand-binding molecule. Affinity tags preferably are antibodies, ligands, binding proteins, receptor proteins, haptens, aptamers, carbohydrates, synthetic polyamides, or oligonucleotides. Preferred binding proteins are DNA binding proteins. Preferred DNA binding proteins
30 are zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs. These motifs can be combined in the same specific binding molecule.

Affinity tags, described in the context of nucleic acid probes, are described by Syvnen *et al.*, *Nucleic Acids Res.*, 14:5037 (1986). Preferred affinity tags include biotin, which can be incorporated into nucleic acids. In the disclosed method, affinity tags incorporated into reporter signals can allow the reporter signals to be captured by, adhered to, or coupled to a substrate. Such capture allows separation of reporter signals from other molecules, simplified washing and handling of reporter signals, and allows automation of all or part of the method.

Zinc fingers can also be used as affinity tags. Properties of zinc fingers, zinc finger motifs, and their interactions, are described by Nardelli *et al.*, *Zinc finger-DNA recognition: analysis of base specificity by site-directed mutagenesis*. *Nucleic Acids Res.*, 20(16):4137-44 (1992), Jamieson *et al.*, *In vitro selection of zinc fingers with altered DNA-binding specificity*. *Biochemistry*, 33(19):5689-95 (1994), Chandrasegaran, S. and J. Smith, *Chimeric restriction enzymes: what is next?* *Biol Chem*, 380(7-8):841-8 (1999), and Smith *et al.*, *A detailed study of the substrate specificity of a chimeric restriction enzyme*. *Nucleic Acids Res.*, 27(2):674-81 (1999).

Capturing reporter signals on a substrate, if desired, may be accomplished in several ways. In one embodiment, affinity docks are adhered or coupled to the substrate. Affinity docks are compounds or moieties that mediate adherence of a reporter signal by associating or interacting with an affinity tag on the reporter signal. Affinity docks immobilized on a substrate allow capture of the reporter signals on the substrate. Such capture provides a convenient means of washing away molecules that might interfere with subsequent steps. Captured reporter signals can also be released from the substrate. This can be accomplished by dissociating the affinity tag or by breaking a photocleavable linkage between the reporter signal and the substrate.

Substrates for use in the disclosed method can include any solid material to which reporter signals can be adhered or coupled. Examples of substrates include, but are not limited to, materials such as acrylamide, cellulose, nitrocellulose, glass, silicon, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Substrates can have any useful form including thin films or membranes, beads,

bottles, dishes, fibers, optical fibers, woven fibers, shaped polymers, particles, compact disks, and microparticles.

R. Vectors and Expression Sequences

Gene transfer can be obtained using direct transfer of genetic material, in but
5 not limited to, plasmids, viral vectors, viral nucleic acids, phage nucleic acids, phages, cosmids, and artificial chromosomes, or via transfer of genetic material in cells or carriers such as cationic liposomes. Such methods are well known in the art and readily adaptable for use in the method described herein. Transfer vectors can be any nucleotide construction used to deliver genes into cells (e.g., a plasmid), or as part of a
10 general strategy to deliver genes, e.g., as part of recombinant retrovirus or adenovirus (Ram et al. Cancer Res. 53:83-88, (1993)). Appropriate means for transfection, including viral vectors, chemical transfectants, or physico-mechanical methods such as electroporation and direct diffusion of DNA, are described by, for example, Wolff, J. A., et al., Science, 247, 1465-1468, (1990); and Wolff, J. A. Nature, 352, 815-818,
15 (1991).

As used herein, plasmid or viral vectors are agents that transport the gene into the cell without degradation and include a promoter yielding expression of the gene in the cells into which it is delivered. In a preferred embodiment vectors are derived from either a virus or a retrovirus. Preferred viral vectors are Adenovirus, Adeno-
20 associated virus, Herpes virus, Vaccinia virus, Polio virus, AIDS virus, neuronal trophic virus, Sindbis and other RNA viruses, including these viruses with the HIV backbone. Also preferred are any viral families which share the properties of these viruses which make them suitable for use as vectors. Preferred retroviruses include Murine Maloney Leukemia virus, MMLV, and retroviruses that express the desirable
25 properties of MMLV as a vector. Retroviral vectors are able to carry a larger genetic payload, i.e., a transgene or marker gene, than other viral vectors, and for this reason are a commonly used vector. However, they are not useful in non-proliferating cells. Adenovirus vectors are relatively stable and easy to work with, have high titers, and can be delivered in aerosol formulation, and can transfect non-dividing cells. Pox viral
30 vectors are large and have several sites for inserting genes, they are thermostable and can be stored at room temperature. A preferred embodiment is a viral vector which has been engineered so as to suppress the immune response of the host organism, elicited

detection systems to increase the resolution of the detection of molecules having very similar properties. The method involves at least two stages. The first stage is filtration or selection that allows passage or selection of labeled proteins (that is, a subset of the molecules present), based upon intrinsic properties of the reporter signals (and the attached proteins), and discrimination against all other molecules. The subsequent stage(s) further separate(s) and/or detect(s) the labeled proteins that were filtered in the first stage. A key facet of this method is that a multiplexed set of labeled proteins will be selected by the filter and the attached reporter signals subsequently will be cleaved, decomposed, reacted, or otherwise modified to realize the identities and/or quantities of the fragmented reporter signals and/or the fragmented labeled proteins in further stages. There is a correspondence between the reporter signal and the detected daughter fragment(s).

In some embodiments, the disclosed method allows a complex sample of proteins and/or peptides to be quickly and easily cataloged in a reproducible manner. Such a catalog can be compared with other, similarly prepared catalogs of other protein samples to allow convenient detection of differences between the samples. The catalogs, which incorporate a significant amount of information about the protein samples, can serve as fingerprints of the samples which can be used both for detection of related protein samples and comparison of protein samples. For example, the presence or identity of specific organisms can be detected by producing a catalog of proteins and/or peptides of the test organism and comparing the resulting catalog with reference catalogs prepared from known organisms. Changes and differences in protein expression patterns can also be detected by preparing catalogs of proteins from different cell samples and comparing the catalogs. Comparison of protein catalogs produced with the disclosed method is facilitated by the fine resolution that can be provided with, for example, mass spectrometry.

Each labeled protein processed in the disclosed method will result in a signal based on the characteristics of the labeled protein (for example, the mass-to-charge ratio). A complex protein sample can produce a unique pattern of signals. It is this pattern that can allow unique cataloging of protein samples and sensitive and powerful comparisons of the patterns of signals produced from different protein samples.

The presence, amount, presence and amount, or absence of different labeled proteins forms a pattern of signals that provides a signature or fingerprint of the proteins, and thus of the protein sample based on the presence or absence of specific proteins, peptides or protein fragments in the sample. For this reason, cataloging of this pattern of signals (that is, the pattern of the presence, amount, presence and amount, or absence of labeled proteins) is an embodiment of the disclosed method that is of particular interest.

Catalogs can be made up of, or be referred to, as, for example, a pattern of labeled proteins, a pattern of the presence of labeled proteins, a catalog of labeled proteins, a catalog of proteins in a sample, or a catalog of amino acid sequences in a sample. The information in the catalog is preferably in the form of mass-to-charge information, compositional information (that is, the composition of amino acids) or, more preferably, in the form of amino acid sequences. Catalogs can also contain or be made up of other information derived from the information generated in the disclosed method (for example, the identity of the proteins detected), and can be combined with information obtained or generated from any other source. The informational nature of catalogs produced using the disclosed method lends itself to combination and/or analysis using known bioinformatics systems and methods.

Such catalogs of protein samples can be compared to a similar catalog derived from any other sample to detect similarities and differences in the samples (which is indicative of similarities and differences in the proteins in the samples). For example, a catalog of a first protein sample can be compared to a catalog of a sample from the same type of organism as the first protein sample, a sample from the same type of tissue as the first protein sample, a sample from the same organism as the first protein sample, a sample obtained from the same source but at time different from that of the first protein sample, a sample from an organism different from that of the first protein sample, a sample from a type of tissue different from that of the first protein sample, a sample from a strain of organism different from that of the first protein sample, a sample from a species of organism different from that of the first protein sample, or a sample from a type of organism different from that of the first protein sample.

When comparing catalogs of proteins obtained from related samples, it is possible to identify the presence of a subset of correlated pairs of labeled proteins and

their altered forms. The disclosed method can be used to detect the original labeled proteins (and determine characteristics of them) and the altered form of the labeled proteins. This pair of detected proteins will be characteristic of the protein that is labeled and the specific reporter signal used (although not necessarily unique).

5 C. Reporter Signal Calibration

In another form of the method, referred to as reporter signal calibration (RSC), a form of reporter signals referred to as reporter signal calibrators are mixed with analytes or analyte fragments, the reporter signal calibrators and the analytes or analyte fragments are altered, and the altered forms of the reporter signal calibrators and altered
10 forms of the analytes or analyte fragments are detected. Reporter signal calibrators are useful as standards for assessing the amount of analytes present. That is, one can add a known amount of a reporter signal calibrator in order to assess the amount of analyte present comparing the amount of altered analyte or analyte fragment detected with the amount of altered reporter signal calibrator detected and calibrating these amounts with
15 the known amount of reporter signal calibrator added (and thus the predicted amount of altered reporter signal calibrator).

The disclosed reporter signal calibration method generates, with high sensitivity, unique analyte signatures related to the relative abundance of different analytes in tissue, microorganisms, or any other biological sample. The disclosed
20 method allows one, for example, to define the status of a cell or tissue by identifying and measuring the relative concentrations of a small but highly informative subset of analytes. Such as measurement is known as an analyte signature. Analyte signatures are useful, for example, in the diagnosis, grading, and staging of cancer, in drug screening, and in toxicity testing.

25 In some embodiments, each analyte or analyte fragment can share one or more common properties with at least one reporter signal calibrator such that the reporter signal calibrators and analytes or analyte fragments having the common property can be distinguished and/or separated from other molecules lacking the common property.

In some embodiments, reporter signal calibrators and analytes and analyte
30 fragments can be altered such that the altered form of an analyte or analyte fragment can be distinguished from the altered form of the reporter signal calibrator with which the analyte or analyte fragment shares a common property. In some embodiments, the

altered forms of different reporter signal calibrators can be distinguished from each other. In some embodiments, the altered forms of different analytes or analyte fragments can be distinguished from each other.

5 In some embodiments of reporter signal calibration, the analyte or analyte fragment is not altered and so the altered reporter signal calibrators and the analytes or analyte fragments are detected. In this case, the analyte or analyte fragment can be distinguished from the altered form of the reporter signal calibrator with which the analyte or analyte fragment shares a common property.

10 In some embodiments the analyte or analyte fragment may be a reporter signal or a fragment of a reporter signal. In this case, the reporter signal calibrators can serve as calibrators for the amount of reporter signal detected.

Reporter signal calibration is preferably used in connection with proteins and peptides (as the analytes). This form of reporter signal calibration is referred to as reporter signal protein calibration. Reporter signal protein calibration is useful, for
15 example, for producing protein signatures of protein samples. As used herein, a protein signature is the presence, absence, amount, or presence and amount of a set of proteins or protein surrogates.

In some embodiments of reporter signal protein calibration, the presence of labile, scissile, or cleavable bonds in the proteins to be detected can be exploited.
20 Peptides, proteins, or protein fragments (collectively referred to, for convenience, as protein fragments in the remaining description) containing such bonds can be altered by fragmentation at the bond. In this way, reporter signal calibrators having a common property (such as mass-to-charge ratio) with the protein fragments can be used and the altered forms of the reporter signal calibrators and the altered (that is, fragmented)
25 forms of the protein fragments can be detected and distinguished. In this regard, although the protein fragments share a common property with their matching reporter signal calibrators, the altered forms of the reporter signal calibrators and altered forms of protein fragments can be distinguished (because, for example, the altered forms have different properties, such as different mass-to-charge ratios).

30 The disclosed reporter signal protein calibration method generates, with high sensitivity, unique protein signatures related to the relative abundance of different proteins in tissue, microorganisms, or any other biological sample. The disclosed

method allows one, for example, to define the status of a cell or tissue by identifying and measuring the relative concentrations of a small but highly informative subset of proteins. Such as measurement is known as a protein signature. Protein signatures are useful, for example, in the diagnosis, grading, and staging of cancer, in drug screening, and in toxicity testing.

As an example of reporter signal protein calibration, a protein sample of interest can be digested with a serine protease, preferably trypsin. The digest generates a complex mixture of protein fragments. Among these protein fragments, there will exist a subset (approximately one protein fragment among every 400) that contains the dipeptide Asp-Pro. This dipeptide sequence is uniquely sensitive to fragmentation during mass spectrometry, and thus produces a high yield of ions in fragmentation mode. Since the human proteome consists of at least 2,000,000 distinct tryptic peptides, the number of protein fragments containing the Asp-Pro sequence is of the order of 5,000. Since some of these may exist as phosphopeptides or other modified forms, the number may be even higher. This number is sufficiently high to permit the selection of a subset (perhaps 50 to 100 or so) of fragmentable protein fragments that is suitable for generating a highly informative protein signature. Peptides that contain the Asp-Pro dipeptide sequence, peptides that contain amino acids that are modified by phosphorylation inside the cell, or peptides that contain an internal methionine are particularly preferred for use in reporter signal calibration. Alternatively, tryptic peptides terminating in arginine may be modified by reaction with acetylacetone (pentane-2,4-dione) to increase the frequency of fragment ions (Dikler et al., J Mass Spectrom 32:1337-49 (1997)). Selection of the subsets of protein fragments can be performed using bioinformatics in order to maximize the information content of the protein signatures.

For this form of reporter signal calibration, the protein digest can be mixed with a specially designed set of reporter signal calibrators, and then can be analyzed using tandem mass spectrometry. In the case of a tandem in space instrument (for example, Q-ToF™ from Micromass), using first quadrupole settings for single-ion filtering (defined by the molecular mass of each unique fragment, which can be obtained from sequence data), followed by a collision stage for ion fragmentation, and finally TOF spectrometry of the peptide fragments (generated by cleavage at fragile bonds, such as

Asp-Pro, bonds involving a phosphorylated amino-acid, or bonds adjacent to an oxidized amino-acid such as methionine sulfoxide, Smith et al., Free Radic Res. 26:103-11 (1997)) that arise from the original single-ion. In the second stage, signal to noise of the TOF measurement is much larger than in a conventional MS experiment.

5 In general, one reporter signal calibrator can be used for each protein fragment in the sample that will be used to make up the protein signature (such protein fragments are referred to as signature protein fragments), and each is designed to fragment in an easily detectable pattern of masses, distinct from the fragment masses of the unfragmented signature protein fragments. The quadrupole filtering settings are then
10 varied in sequence over a range of values (fifty, for example), corresponding to the masses of each of the protein fragments previously chosen to comprise the protein signature (that is, the signature protein fragments). At each filtered mass setting, there will be two types of signals detectable by TOF after fragmentation, one set derived from the tryptic peptide (that is, the original protein fragment), and another set
15 corresponding to the reporter signal calibrator. The reporter signal calibrator permits one to calculate relative abundance for each of the signature protein fragments. These relative abundance ratios, determined for a given sample, constitute the protein signature. The detection limit of the tandem mass spectrometer in MS/MS mode, is remarkably good, perhaps of the order of 500 molecules of peptide. This level of
20 detection is approximately 1,000 times better than that for MALDI-TOF mass spectrometry, and should permit the generation of protein signatures from single cells.

As can be seen, for this form of reporter signal calibration, the availability of the sequence of the entire human genome, as well as the genomes of many other organisms, can facilitate the identification of protein fragments that are unique in the
25 context of all known proteins. That is, the sequence information can be used to identify peptides that will be generated in a protein signature and guide selection of reporter signal calibrators.

D. Reporter Signal Fusions

In another form of the disclosed method, referred to as reporter signal fusions
30 (RSF), reporter signal peptides are joined with a protein or peptide of interest in a single amino acid segment, and the reporter signal peptide, reporter signal fusion,

altered forms of the reporter signal peptide, and/or altered forms of the reporter signal fusion can be detected.

The disclosed method provides sensitive monitoring and detection of the proteins and peptides to which reporter signal peptides are fused. In particular, the method provides sensitive and multiplex detection of expression of particular proteins and peptides of interest, and/or of the genes, vectors, and expression constructs encoding the proteins and peptides of interest.

The disclosed method can use cells, cell lines, and organisms that have particular protein(s), gene(s), vector(s), and/or expression sequence(s) labeled (that is, associated with or involved in) reporter signal fusions. For example, a set of nucleic acid constructs, each encoding a reporter signal fusion with a different reporter signal peptide, can be used to uniquely label a set of cells, cell lines, and/or organisms. Processing, in the disclosed method, of a sample from any of the labeled sources can result in a unique altered form of the reporter signal peptide (or the amino acid segment or an amino acid subsegment) for each of the possible sources that can be distinguished from the other altered forms. Detection of a particular altered form identifies the source from which it came. As a more specific example, a genetically modified plant line (for example, a Roundup resistant corn line) into which a nucleic acid construct encoding a reporter signal fusion has been introduced can be identified by detecting the reporter signal fusion.

The disclosed method can also be used to assess the state and/or expression of particular pathways, regulatory cascades, and other suites of genes, proteins, vectors, and/or expressions sequences. By using reporter signal fusions to "label" such pathways, cascades, etc. within the same cell, cell line, or organism, or across a set of cells, cell lines, or organisms, the pathways, cascades and other systems can be assessed in a single assay and/or compared across cells, cell lines, or organisms. For example, nucleic acid segments encoding reporter signal fusions can be associated with endogenous expression sequences of interest, endogenous genes of interest, exogenous expression sequences of interest, exogenous genes of interest, or a combination, and the expression of the genes and/or expression sequences assessed by detecting the reporter signal peptides and/or reporter signal fusions. Many other modes of the method are possible, a number of which are described in the illustrations below. In particular, the

disclosed reporter signal fusions can be used in the disclosed method for purposes analogous to any purpose that green fluorescent protein and green fluorescent protein fusions are used. However, the disclosed method can make use of reporter signal fusions in many more ways and for more useful purposes than green fluorescent protein at least because of the ability to multiplex the disclosed reporter signal fusions.

Nucleic acid sequences encoding reporter signal peptides can be engineered into particular exons of a gene. This would be the normal situation when the gene encoding the protein to be fused contains introns, although sequence encoding a reporter signal peptide can be split between different exons to be spliced together. Placement of nucleic acid sequences encoding reporter signal peptides into particular exons is useful for monitoring and analyzing alternative splicing of RNA. The appearance of a reporter signal peptide in the final protein indicates that the exon encoding the reporter signal peptide was spliced into the mRNA.

The disclosed method can provide sensitive detection of one or multiple proteins (that is, proteins to which reporter signal peptides are fused). In the method, proteins fused with reporter signal peptides are analyzed using the reporter signal peptides to distinguish the reporter signal fusions. Detection of the reporter signal peptides indicates the presence of the corresponding protein(s). The detected protein(s) can then be analyzed using known techniques. The reporter signal fusions provide a unique protein/label composition that can specifically identify the protein(s). This is accomplished through the use of the specialized reporter signal peptides as the labels.

In the method, reporter signal fusions can be fragmented, such as by protease digestion, prior to analysis. An expression sample to be analyzed can also be subjected to fractionation or separation to reduce the complexity of the samples. Fragmentation and fractionation can also be used together in the same assay. Such fragmentation and fractionation can simplify and extend the analysis of the expression.

In the method, reporter signal peptides can be fragmented, decomposed, reacted, derivatized, or otherwise modified, preferably in a characteristic way. This allows a protein to which the reporter signal peptide is fused to be identified by detection of one or more of the products of the reporter signal fusion following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal peptide. The protein can also be identified by the correlated detection of the reporter

signal fusion and one or more of the products of the reporter signal fusion following fragmentation, decomposition, reaction, derivatization, or other modification of the reporter signal peptide. The alteration of the reporter signal peptide will alter the reporter signal fusion in a characteristic and detectable way. Together, the detection of
5 a characteristic reporter signal fusion and a characteristic product of (that is, altered form of) the reporter signal fusion can uniquely identify the protein (although the altered form alone can be detected, if desired). In this way, using the disclosed method, expression of one or more proteins can be detected, either alone or together (for example, in a multiplex assay). Further, expression of one or more proteins in one or
10 more samples can be detected in a multiplex manner. Preferably, for mass spectrometry reporter signals, the reporter signal peptides are fragmented to yield fragments of similar charge but different mass.

Preferably, the reporter signal peptides are fragmented to yield fragments of similar charge but different mass. This allows each reporter signal fusion (and/or each
15 reporter signal peptide) in a set to be distinguished by the different mass-to-charge ratios of the fragments of (that is, altered forms of) the reporter signal peptides. This is possible since the fragments of the different reporter signal peptides (or the fragments of the reporter signal fusions) can be designed to have different mass-to-charge ratios. In the disclosed method, this allows each reporter signal fusion to be distinguished by
20 the mass-to-charge ratios of the reporter signal fusions after fragmentation of the reporter signal peptide.

Alteration of reporter signals peptides in reporter signal fusions can produce a variety of altered compositions. Any or all of these altered forms can be detected. For example, the altered form of the reporter signal peptide can be detected, the altered
25 form of the amino acid segment (which contains the reporter signal peptide) can be detected, the altered form of a subsegment of the amino acid segment can be detected, or a combination of these can be detected. Where the reporter signal peptide is altered by fragmentation, the result generally will be a fragment of the reporter signal peptide and an altered form of the amino acid segment containing the protein or peptide of
30 interest and a portion of the reporter signal peptide (that is, the portion not in the reporter signal peptide fragment).

The protein or peptide of interest also can be fragmented. The result would be a subsegment of the amino acid segment. The amino acid subsegment would contain the reporter signal peptide and a portion of the protein or peptide of interest. When the reporter signal peptide in an amino acid subsegment is altered (which can occur before, during, or after fragmentation of the amino acid segment), the result is an altered form of the amino acid subsegment (and an altered form of the reporter signal peptide). This altered form of amino acid subsegment can be detected. Where the reporter signal peptide is altered by fragmentation, the result generally will be a fragment of the reporter signal peptide and an altered form of (that is, fragment of) the amino acid subsegment. In this case, the altered form of the amino acid subsegment will contain a portion of the protein or peptide of interest and a portion of the reporter signal peptide (that is, the portion not in the reporter signal peptide fragment).

Cells, cell lines, organisms, and expression of genes and proteins can be detected using the disclosed reporter signal fusions in a variety of ways. For example, the protein and attached reporter signal peptide can be detected together, one or more peptides of the protein and the attached reporter signal peptide(s) can be detected together, the fragments of the reporter signal peptide can be detected, or a combination. Preferred detection involves detection of the reporter signal fusion both before and after fragmentation of the reporter signal peptide.

A preferred form of the disclosed method involves correlated detection of the reporter signal peptides both before and after fragmentation of the reporter signal peptide. This allows genes, proteins, vectors, and expression constructs "labeled" with a reporter signal peptide to be detected and identified via the change in the reporter signal fusion and/or reporter signal peptide. That is, the nature of the reporter signal fusion or reporter signal peptide detected (non-fragmented versus fragmented) identifies the gene, protein, vector, or nucleic acid construct from which it was derived. Where the reporter signal fusions and reporter signal peptides are detected by mass-to-charge ratio, the change in mass-to-charge ratio between fragmented and non-fragmented samples provides the basis for comparison. Such mass-to-charge ratio detection is preferably accomplished with mass spectrometry.

As an example, a fusion between a protein of interest and a reporter signal peptide designed as a mass spectrometry label can be expressed. The reporter signal

fusion can be subjected to tryptic digest followed by mass spectrometry of the resulting materials. A peak corresponding to the tryptic fragment containing the reporter signal peptide will be detected. Fragmentation of the reporter signal peptide in a collision cell in the mass spectrometer would result in a shift in the peak corresponding to the loss of a portion of the attached reporter signal peptide, the appearance of a peak
5 corresponding to the lost fragment, or a combination of both events. Significantly, the shift observed will depend on which reporter signal peptide is fused to the protein since different reporter signal peptides will, by design, produce fragments with different mass-to-charge ratios. The combination event of detection of the parent mass-to-charge
10 (with no collision gas) and the mass-to-charge corresponding to the loss of the fragment from the reporter signal peptide (with collision gas) indicates a reporter signal fusion (thus indicating expression of the reporter signal fusion and of the gene, vector, or construct encoding it).

A powerful form of the disclosed method is use of reporter signal fusions to
15 assay multiple samples (for example, time series assays or other comparative analyses). Knowledge of the temporal response of a biological system following perturbation is a very powerful process in the pursuit of understanding the system. To follow the temporal response a sample of the system is obtained (for example, cells from a cell culture, mice initially synchronized and sacrificed) at determined times following the
20 perturbation. Knowledge of spatial protein profiles (for example, relative position within a tissue section) is a very powerful process in the pursuit of understanding the biological system.

The reporter signal fusions are preferably detected using mass spectrometry which allows sensitive distinctions between molecules based on their mass-to-charge
25 ratios. A set of isobaric reporter signal peptides or reporter signal fusions can be used for multiplex labeling and/or detection of the expression of many genes, proteins, vectors, expression constructs, cells, cell lines, and organisms since the reporter signal peptide fragments can be designed to have a large range of masses, with each mass individually distinguishable upon detection. Where the same gene, protein, vectors,
30 expression construct, cell, cell line, or organism (or the same type of gene, protein, vector, expression construct, cell, cell line, or organism) is labeled with a set of reporter signal fusions that are isobaric or that include isobaric reporter signal peptides (by, for

example, "labeling" the same gene, protein, vector, expression construct, cell, cell line, or organism in different samples), the set of reporter signal fusions or reporter signal peptides that results will also be isobaric. Fragmentation of the reporter signal peptides will split the set of reporter signal peptides into individually detectable reporter signal fusion fragments and reporter signal peptide fragments of characteristically different mass.

A preferred form of the disclosed method involves filtering of isobaric reporter signal fusions or reporter signal peptides from other molecules based on mass-to-charge ratio, fragmentation of the reporter signal peptides to produce fragments having different masses, and detection of the different fragments based on their mass-to-charge ratios. The method is best carried out using a tandem mass spectrometer as described elsewhere herein.

Nucleic acid sequences and segments encoding reporter signal fusions can be expressed in any suitable manner. For example, the disclosed nucleic acid sequences and nucleic acid segments can be expressed *in vitro*, in cells, and/or in cells in organism. Many techniques and systems for expression of nucleic acid sequences and proteins are known and can be used with the disclosed reporter signal fusions. For example, many expression sequences, vector systems, transformation and transfection techniques, and transgenic organism production methods are known and can be used with the disclosed reporter signal peptide method and compositions.

For example, kits for the *in vitro* transcription/translation of DNA constructs containing promoters and nucleic acid sequence to be transcribed and translated are known (for example, PROTEINscript-PRO™ from Ambion, Inc. Austin TX; Wilkinson (1999) "Cell-Free And Happy: In Vitro Translation And Transcription/Translation Systems", The Scientist 13[13]:15, Jun. 21, 1999). Such constructs can be used in the genomic DNA of an organism, in a plasmid or other vector that may be transfected into an organism, or in *in vitro* systems. For example, constructs containing a promoter sequence and a nucleic acid sequence that, following transcription and translation, results in production of green fluorescence protein or luciferase as a reporter/marker in *in vivo* systems are known (for example, Sawin and Nurse, "Identification of fission yeast nuclear markers using random polypeptide fusions with green fluorescent protein." Proc Natl Acad Sci U S A 93(26):15146-51

(1996); Chatterjee et al., "In vivo analysis of nuclear protein traffic in mammalian cells." *Exp Cell Res* 236(1):346-50 (1997); Patterson et al., "Quantitative imaging of TATA-binding protein in living yeast cells." *Yeast* 14(9):813-25 (1998); Dhandayuthapani et al., "Green fluorescent protein as a marker for gene expression and cell biology of mycobacterial interactions with macrophages." *Mol Microbiol* 17(5):901-12 (1995); Kremer et al., "Green fluorescent protein as a new expression marker in mycobacteria." *Mol Microbiol* 17(5):913-22 (1995); Reiländer et al., "Functional expression of the *Aequorea victoria* green fluorescent protein in insect cells using the baculovirus expression system." *Biochem Biophys Res Commun* 219(1):14-20 (1996); Mankertz et al., "Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma" *J Cell Sci*, 113:2085-90 (2000); White et al., "Real-time analysis of the transcriptional regulation of HIV and hCMV promoters in single mammalian cells" *J Cell Sci*, 108:441-55 (1995)). Green fluorescence protein, or variants, have been shown to be stably incorporated and not interfere with the organism — generally GFP is larger relative to the disclosed reporter signal peptides (GFP from *Aequorea Victoria* is 238 amino acids in size; NCBI GI:606384), and thus the reporter signal peptides are less likely to disrupt an expression system to which they are added.

Techniques are known for modifying promoter regions such that the endogenous promoter is replaced with a promoter-reporter construct, for example, where the reporter is green fluorescent protein (Patterson et al., "Quantitative imaging of TATA-binding protein in living yeast cells." *Yeast* 14(9): 813-25 (1998)) or luciferase. Transcription factor concentrations are followed by monitoring the GFP or luciferase. These techniques can be used with the disclosed reporter signal fusions and reporter signal fusion constructs. Techniques are also known for targeted knock-in of nucleic acid sequences into a gene of interest, typically under control of the endogenous promoter. Such techniques, which can be used with the disclosed method and compositions, have been used to introduce reporter/markers of the transcription and translation of the gene where then nucleic acid was inserted. The same techniques can be used to place the disclosed reporter signal fusions under control of endogenous expression sequences. Alternately, non-targeted knock-ins (techniques for which are also known) can be used to follow the level or activity of transcription factors.

As with reporter signals generally, reporter signal peptides can be used in sets where the reporter signal peptides in a set can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. In the case of reporter signal fusions, amino acid
5 segments and amino acid subsegments can be used in sets where the amino acid segments and amino acid subsegments in a set can have one or more common properties that allow the amino acid segments and amino acid subsegments, respectively, to be separated or distinguished from molecules lacking the common property. In general, the component(s) of the reporter signal fusions having common
10 properties can depend on the component(s) to be detected and/or the mode of the method being used.

Nucleic acid molecules encoding reporter signal fusions can be used in sets where the reporter signal peptides in the reporter signal fusions encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter
15 signal peptides to be separated or distinguished from molecules lacking the common property. Similarly, nucleic acid molecules encoding amino acid segments can be used in sets where the reporter signal peptides in the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the
20 common property. Nucleic acid molecules encoding amino acid segments can be used in sets where the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the amino acid segments to be separated or distinguished from molecules lacking the common property.

Nucleic acid segments (which, generally, are part of nucleic acid molecules)
25 encoding reporter signal fusions can be used in sets where the reporter signal peptides in the reporter signal fusions encoded by a set of nucleic acid segments can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Similarly, nucleic acid segments encoding amino acid segments can be used in sets where the reporter signal
30 peptides in the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the reporter signal peptides to be separated or distinguished from molecules lacking the common property. Nucleic acid

segments encoding amino acid segments can be used in sets where the amino acid segments encoded by a set of nucleic acid molecules can have one or more common properties that allow the amino acid segments to be separated or distinguished from molecules lacking the common property. Other relationships between members of the sets of nucleic acid molecules, nucleic acid segments, amino acid segments, reporter signal peptides, and proteins of interest are contemplated.

Reporter signal fusions can include other components besides a protein of interest and a reporter signal peptide. For example, reporter signal fusions can include epitope tags or flag peptides. Epitope tags and flag peptides can serve as tags by which reporter signal fusions can be separated, distinguished, associated, and/or bound. The use of epitope tags and flag peptides generally is known and can be adapted for use in the disclosed reporter signal fusions.

In preferred embodiments, reporter signal peptides, reporter signal fusions (or amino acid segments), nucleic acid segments encoding reporter signal fusion, and/or nucleic acid molecules comprising nucleic acid segments encoding reporter signal fusions are used in sets where the reporter signal peptides, the reporter signal fusions, and/or subsegments of the reporter signal fusions constituting or present in the set have similar properties (such as similar mass-to-charge ratios). The similar properties allow the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions to be distinguished and/or separated from other molecules lacking one or more of the properties. Preferably, the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions constituting or present in a set have the same mass-to-charge ratio (m/z). That is, the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions in a set are isobaric. This allows the reporter signals, the reporter signal fusions, or subsegments of the reporter signal fusions to be separated precisely from other molecules based on mass-to-charge ratio. The result of the filtering is a huge increase in the signal to noise ratio (S/N) for the system, allowing more sensitive and accurate detection.

Preferred reporter signal peptides for use in reporter signal fusions used in or associated with different genes, proteins, vectors, constructs, cells, cell lines, or organisms would be those using differentially distributed mass. In particular, the use of alternative amino acid sequences using the same amino acid composition is preferred.

Although reference is made above and elsewhere herein to detection of, and fusion with, a "protein" or "proteins," the disclosed method can involve proteins, peptides, and fragments of proteins or peptides. Thus, reference to a protein herein is intended to refer to proteins, peptides, and fragments of proteins or peptides unless the context clearly indicates otherwise. As used herein "reporter signal fusion" refers to a protein, peptide, or fragment of a protein or peptide to which a reporter signal peptide is fused (that is, joined by peptide bond(s) in the same polypeptide chain) unless the context clearly indicates otherwise. The reporter signal peptide(s) can be fused to a protein in any arrangement, such as at the N-terminal end of the protein, at the C-terminal end of the protein, in or at domain junctions, or at any other appropriate location in the protein. In some forms of the method, it is desirable that the protein remain functional. In such cases, terminal fusions or inter-domain fusions are preferably. Those of skill in the art of protein fusions generally know how to design fusions where the protein of interest remains functional. In other embodiments, it is not necessary that the protein remain functional in which case the reporter signal peptide and protein can have any desired structural organization.

The reporter signal fusions can be produced by expression from nucleic acid molecules encoding the fusions. Thus, the disclosed fusions generally can be designed by designing nucleic acid segments that encode amino acid segments where the amino acid segments comprise a reporter signal peptide and a protein or peptide of interest. A given nucleic acid molecule can comprise one or more nucleic acid segments. A given nucleic acid segment can encode one or more amino acid segments. A given amino acid segment can include one or more reporter signal peptides and one or more proteins or peptides of interest. The disclosed amino acid segments consist of a single, contiguous polypeptide chain. Thus, although multiple amino acid segments can be part of the same contiguous polypeptide chain, all of the components (that is, the reporter signal peptide(s) and protein(s) and peptide(s) of interest) of a given amino acid segment are part of the same contiguous polypeptide chain.

Reporter signal fusions can be used to monitor and analyze alternative RNA splicing. A central problem in translating the information in the genome to protein expression is an understanding of mRNA alternative processing, and the generation of protein isoforms via alternative exon utilization (Black, "Protein diversity from

alternative splicing: a challenge for bioinformatics and post-genome biology" *Cell* 103:367-70 (2000)). Many examples of the use of alternative pre-mRNA splicing to generate protein isoform diversity exist, such as in the control of erythroid differentiation (see, for example, Hou and Conboy, "Regulation of alternative pre-mRNA splicing during erythroid differentiation" *Curr Opin Hematol* 8:74-9 (2001)).

Often the detection of complex, alternatively spliced protein isoforms is a difficult task, since exons may be as small as 6 amino acids in protein of over 2000 amino acids (see, for example, Cianci et al., "Brain and muscle express a unique alternative transcript of α II spectrin" *Biochem* 38:15721-15730 (1999)).

- 10 Exon utilization and processing information can be obtained by insertion of a nucleic acid sequence encoding a reporter signal into the exon sequence of interest (thus forming a nucleic acid segment that encodes a reporter signal fusion). The insertions can be made, for example, into genomic DNA, appropriate mini-gene constructs, or non-endogenous pre-mRNA introduced into the cell. Use of a set of
- 15 reporter signals allows the multiplexed readout of all exons of a translated protein at one time. The use of mini-gene constructs or constructs incorporating short exogenous open-reading frame DNA sequences into exons, and the incorporation of foreign DNA in association with functional intron splice elements are developed technologies that can be used for incorporation of reporter signals (see, for example, Gee et al.,
- 20 "Alternative splicing of protein 4.1R exon 16: ordered excision of flanking introns ensures proper splice site choice" *Blood* 95:692-9 (2000); Kikumori et al., "Promiscuity of pre-mRNA spliceosome-mediated trans splicing: a problem for gene therapy?" *Hum Gene Ther* 12:1429-41 (2001); Malik et al., "Effects of a second intron on recombinant MFG retroviral vector" *Arch Virol* 146:601-9 (2001); Virts and
- 25 Raschke, "The role of intron sequences in high level expression from CD45 cDNA constructs" *J Biol Chem* 276:19913-20 (2001)). Detection of the reporter signals, the amounts of the reporter signals, and the knowledge of which reporter signal correlates with which exon, provides information about exon usage and alternative splicing.

- The disclosed reporter signal fusions also can be used in the detection and
- 30 analysis of protein interactions with other proteins and molecules. For example, interaction traps for protein-protein interactions include the well known yeast two-hybrid (Fields and Song. "A novel genetic system to detect protein-protein interactions"

Nature 340:245-6 (1989); Uetz et al., "A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*" Nature 403:623-7 (2000)) and related systems (Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 2001; Van Crieginge and Beyaert, "Yeast two-hybrid: state of the art" Biological Procedures Online, 2(1), 1999). Incorporation of nucleic acid sequence encoding a peptide reporter signal can be introduced into these systems, for example at a terminus of the ordinarily used LacZ selection region (LacZ selection is described in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, second edition, 1989, Cold Spring Harbor Laboratory Press, New York). A set of such incorporated sequences (for example, in a set of such plasmids, where each plasmid has a reporter signal coding sequence and the LacZ functionality), allows the unambiguous detection of many interactions simultaneously rather (as many different interactions as reporter signals used).

In another mode of reporter signal fusions, a nucleic acid sequence encoding a reporter signal could be added to sequence encoding the constant (C) region of T cell and B cell receptors. The reporter signal would appear in T or B cell receptors when that C region is spliced to a J region following transcription.

In another mode of reporter signal fusions, referred to as reporter signal presentation, the presentation of specific antigenic peptides by major histocompatibility (MHC) and non-major histocompatibility molecules can be detected and analyzed. It is well known that protein antigens are processed by antigen presenting cells and that small peptides, typically 8-12 amino acids are presented by Class I and Class II MHC molecules for recognition by T cells. The study of specific T cell/peptide-MHC complexes is technically challenging due various labeling requirements (either radioactive or fluorescence) and the common reliance on antibody reagents that recognize specific receptors and/or peptide-MHC complexes.

There is a need to be able to further expand our knowledge of antigen processing and antigen presentation. Reporter signals that have been engineered into specific protein antigens could provide novel insight into this process and enable new experimental approaches. For instance, consider two viral or bacterial proteins, protein A and protein B, that differ by only a few amino acids. It would be useful to know if they are processed and presented to immune cells (for example, T cells) with the same

efficiency. By engineering reporter signals into protein A and engineered protein B to antigen presenting cells, one could test for the presence of the different reporter signals presented on and thus determine if the proteins are efficiently processed and presented. The presence of reporter signal A (present in protein A) but not reporter signal B (present in protein B), indicates that protein A is processed and that protein B is not. The lack of antigen processing of protein B may then be an explanation of why a virus or bacteria escapes immune surveillance by the immune system. Antigenic peptides are characterized by conserved anchor residues near both the amino and carboxy ends, with more heterogeneity tolerated in the middle. This middle heterogeneity is thus a preferred site for addition of a reporter signal peptide.

E. Rearranging Reporter Signals

Another embodiment of the disclosed method and compositions, referred to as rearranging reporter signals, enables one to detect the occurrence of specific gene rearrangement events, their protein products, and specific cell populations bearing those receptors. Rearranging reporter signals will also allow one to follow the progression or development of certain receptors and cells or populations of cells by monitoring the presence and/or absence of a reporter signal. Design considerations for rearranged reporter signals are analogous to those required for reporter signal fusions as described elsewhere herein.

Most embodiments of the disclosed method involve intact reporter signals that are associated with analytes in various ways. Rearranging reporter signals make use of processes, such as biological processes, to form reporter signals by specific rearrangement of the reporter signal pieces or rearrangement of nucleic acid segments encoding only portions of reporter signals. One form of rearranging reporter signals utilizes endogenous biological systems, such as the variable-diversity-joining (V-D-J) gene rearrangement machinery present in the mammalian immune system. In this system, short stretches of germline DNA (the V, D & J gene fragments) that are not contiguous, are brought together (recombined) prior to serving as a template for transcription. Gene rearrangement occurs in white blood cells such as T and B lymphocytes and is a key mechanism for generating diversity of T cell and B cell antigen receptors. Theoretically, billions of different receptors can be generated. This level of complexity makes it difficult to detect the presence of rare rearrangement

events, or receptors. PCR based assays and flow cytometry approaches are now used to study receptor diversity. However, PCR approaches are laborious and do not provide any information on the status of expressed protein. Flow cytometry approaches have limited multiplexing capabilities due to emission spectra overlap of the fluorescent probes used.

If one desired to test for 50-100 T cell or B cell receptors, one would need to make use of a similar number of antibodies to those receptors, something that in practice is not done. Therefore, there is a real need for methods that would allow highly sensitive and specific detection of specific receptors in a highly complex pool of receptors. The ability to highly multiplex this approach would enable currently unattainable experimental approaches. The disclosed reporter signal technology allows large scale multiplexing of signals for detection.

As an example of rearranging reporter signals, transgenic mice can be generated in which nucleic acid sequences encoding reporter signals have been engineered into the mouse germline. Methods for doing this are well known in the art and include using standard molecular biology methods to engineer rearranging reporter signal into, for example, yeast or bacterial artificial chromosomes (YACs or BACs) and then using these constructs to generate transgenic mice.

As an example of the use of immunoglobulin rearrangement for rearranging reporter signals, part of a reporter signal could be encoded on the D region and another part of the reporter signal could be encoded on the J region. Upon a rearrangement event that joined the D and J regions encoding these "partial" reporter signals, a coding sequence for a "complete" reporter signal would be generated. Following transcription and translation, the reporter signal would be encoded within the protein product. The reporter signal could then be detected as described elsewhere herein. In the absence of a rearrangement event that joins the engineered D and J region, no reporter signal would be detected. By including sequences encoding parts of a variety of reporter signals with different D and J regions, a variety of different reporter signals can be generated by rearrangement, a different, and diagnostic, reporter signal for each of the different possible rearrangements. This system also could be extended to include, for example, reporter signals split among three or more gene regions (for example, V-D-J, V-D-D-J, etc) with the result that multiple rearrangement events would produce the

reporter signal. In this mode, the combinations of rearrangements of the reporter signal parts can give rise to an large number of different reporter signals, each characterized by the specific reporter signal parts rearranged to form the reporter signal.

Transgenic mice carrying rearranging reporter signals would enable one to address questions that would otherwise be very difficult or impossible to address. For instance, one could dissect what specific T and B cell receptors (out of the thousands or millions possible) respond to specific stimuli or what cell types are present at certain stages of development.

F. Lipid Reporter Signals

The disclosed method and compositions also can be used to monitor lipid composition, distribution, and processing. Lipids are hydrophobic biomolecules that have high solubility in organic solvents. They have a variety of biological roles that make them valuable targets for monitoring. As a nutritional source, lipids (together with carbohydrates) constitute an important source of cellular energy and metabolic intermediates needed for cell signaling and other processes. Lipids processed for energy conversion typically pass through a variety of enzymatic pathways, generating many intermediates. A summary of these cycles is available in most modern biochemistry texts (see, for example, Stryer, 1995). Monitoring the processing of acyl chain intermediates as they are metabolized is an important tool in lipid and cell biological research, as well as for the clinical detection of biochemical diseases such as medium-chain acyl-CoA dehydrogenase deficiencies (see, for example, Zschocke et al., "Molecular and functional characterization of mild MCAD deficiency.", Hum Genet 108:404-8 (2001)). Incorporating reporter signals into, or associating reporter signals with, lipids can improve methods of detecting lipids (such as Andresen et al., "Medium-chain acyl-CoA dehydrogenase (MCAD) mutations identified by MS/MS-based prospective screening of newborns differ from those observed in patients with clinical symptoms: identification and characterization of a new, prevalent mutation that results in mild MCAD deficiency" Am J Hum Genet 68:1408-18. (2001)) by allowing, for example, more rapid and multiplex detection of processed acyl chain intermediates.

In another role, lipids function as the most fundamental and defining component of all biological membranes. The three major types of membrane lipids are phospholipids, glycolipids, and cholesterol. The most abundant of these are the

phospholipids, derived either from glycerol or sphingosine. Those based on glycerol typically contain two esterified long-chain fatty acids (14 to 24 carbons) and a phosphorylated alcohol or sugar. Phospholipids based on sphingosine contain a single fatty acid. Collectively these lipids contribute to the structure and fluidity of biological membranes. Cyclic changes in their processing, particularly of acidic glycosphospholipids such as phosphatidyl inositol 4,5 phosphate, also regulate a wide variety of cellular processes (see, for example, Cantrell, "Phosphoinositide 3-kinase signaling pathways" *J Cell Sci* 114:1439-45 (2001); Payrastre et al., "Phosphoinositides: key players in cell signaling, in time and space" *Cell Signal* 13:377-87 (2001)). Thus, by incorporating reporter signals into, or associating reporter signals with, the acyl chains of such molecules, the subsequent incorporation of such reporter molecules into either in vitro assays such as those used for enzyme determinations or in vivo assays, allows one to rapidly follow the segregation of these lipids into distinct cellular compartments (for example, golgi versus plasma membrane (see, for example, Godi et al., "ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex" *Nat Cell Biol* 1:280-7 (1999)), and their processing via metabolic and signaling pathways such as those cited above.

It is known that exogenous lipid labels can be incorporated readily into biological systems, and the disclosed reporter signals also can be incorporated into such systems. For example, spin-labeled acyl fatty acids and phospholipids have been incorporated into the membranes of phospholipid vesicles and cells (see, for example, Kornberg and McConnell, "Inside-outside transitions of phospholipids in vesicle membranes" *Biochemistry* 10:1111-20 (1971); Kornberg and McConnell, "Lateral diffusion of phospholipids in a vesicle membrane" *Proc Natl Acad Sci USA* 68:2564-8 (1971); Arora et al., "Selectivity of lipid-protein interactions with trypsinized Na, K-ATPase studied by spin-label EPR" *Biochim Biophys Acta* 1371:163-7 (1998); Alonso et al., "Lipid chain dynamics in stratum corneum studied by spin label electron paramagnetic resonance" *Chem Phys Lipids* 104:101-11 (2000)).

Triglycerides, or the acyl chain of sphingolipids or glycolipids, and cholesterol, may be synthesized to include a reporter signal. An example of such a reporter signal would be a lipid made from an aliphatic chain with a carboxylic acid with a photocleavable bond. Examples of photocleavable bonds are described by Glatthar and

Geise, Org. Lett, 2:2315-2317 (2000); Guillier et al., Chem. Rev. 100:2091-2157 (2000); Wierenga, U.S. Patent No. 4,086,254; and elsewhere here. A set of reporter signals may be prepared by locating the cleavable bond at different locations within an aliphatic chain (thus resulting in fragments of different mass when the bond is cleaved).

- 5 The aliphatic chain with a photocleavable bond constitutes the reporter signal. Such synthetic reporter molecules can be incorporated into synthetic triglycerides by, for example, a dehydration reaction. Once formed, a set of these synthetic triglycerides can be introduced into biological systems of interest, such as those described above. Reporter signals can be recovered from the biological system of interest for detection and quantitation by, for example, extraction of the lipid into chloroform and release of
10 reporter signals from the triglyceride using a lipase or hydrolysis reaction.

Forms and Embodiments of the Disclosed Methods

A. Reporter Molecule Labeling

- Disclosed are methods comprising (a) separating a set of reporter signals, where
15 each reporter signal has a common property, from molecules lacking the common property, (b) altering the reporter signals, (c) detecting and distinguishing the altered forms the reporter signals from each other.

- Also disclosed are methods wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their mass, wherein the altered
20 forms of the reporter signals are distinguished via differences in the mass-to-charge ratio of the altered forms of reporter signals.

Also disclosed are methods wherein the mass of the reporter signals is altered by fragmentation.

- Also disclosed are methods wherein the set of reporter signals comprises two or
25 more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signals.

- Also disclosed are methods wherein the set of reporter signals comprises ten or
30 more different reporter signals.

Also disclosed are methods wherein the reporter signals are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

Also disclosed are methods wherein the reporter signals are associated with, or coupled to, specific binding molecules, wherein each reporter signal is associated with, or coupled to, a different specific binding molecule.

Also disclosed are methods wherein the reporter signals are associated with, or coupled to, decoding tags, wherein each reporter signal is associated with, or coupled to, a different decoding tag.

Also disclosed are methods further comprising, prior to step (a), associating the reporter signals with one or more analytes, wherein each reporter signal is associated with, or coupled to, a different specific binding molecule, wherein each specific binding molecule can interact specifically with a different one of the analytes, wherein the reporter signals are associated with the analytes via interaction of the specific binding molecules with the analytes.

Also disclosed are methods wherein steps (a) through (c) are repeated one or more times using a different set of reporter signals each time.

Also disclosed are methods wherein, prior to step (a), the different sets of reporter signals are associated with different samples.

Also disclosed are methods wherein the different sets of reporter signals each comprise the same reporter signals.

Also disclosed are methods wherein the sets of reporter signals each contain a single reporter signal.

Also disclosed are methods wherein not all of the reporter signals in the set are distinguished and/or separated from molecules lacking the common property, not all of the reporter signals are altered, and not all of the altered forms of the reporter signals are detected at the same time.

Also disclosed are methods wherein all of the reporter signals in the set are distinguished and/or separated from molecules lacking the common property, all of the reporter signals are altered, and all of the altered forms of the reporter signals are detected at different times.

Also disclosed are methods wherein steps (a) through (c) are performed separately for each reporter signal.

Also disclosed are methods wherein the reporter signals comprise peptides, wherein the peptides have the same mass-to-charge ratio and methods wherein the

peptides have the same amino acid composition and methods wherein the peptides have the same amino acid sequence and methods wherein each peptide contains a different distribution of heavy isotopes and methods wherein each peptide has a different amino acid sequence and methods wherein each peptide has a labile or scissile bond in a
5 different location.

Disclosed are methods comprising (a) separating one or more reporter signals, where each reporter signal has a common property, from molecules lacking the common property in each of a plurality of samples, (b) altering the reporter signals, (c) detecting and distinguishing the altered forms the reporter signals from each other.

10 Also disclosed are methods wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their mass, wherein the altered forms of the reporter signals are distinguished via differences in the mass-to-charge ratio of the altered forms of reporter signals.

Also disclosed are methods wherein the mass of the reporter signals is altered
15 by fragmentation.

Also disclosed are methods wherein the reporter signals are associated with, or coupled to, specific binding molecules, wherein each reporter signal is associated with, or coupled to, a different specific binding molecule.

Also disclosed are methods wherein the reporter signals are associated with, or
20 coupled to, decoding tags, wherein each reporter signal is associated with, or coupled to, a different decoding tag.

Also disclosed are methods further comprising, prior to step (a), associating the reporter signals with one or more analytes, wherein each reporter signal is associated with, or coupled to, a different specific binding molecule, wherein each specific
25 binding molecule can interact specifically with a different one of the analytes, wherein the reporter signals are associated with the analytes via interaction of the specific binding molecules with the analytes.

Also disclosed are methods wherein steps (a) through (c) are repeated one or more times using a different set of one or more reporter signals each time and methods
30 wherein, prior to step (a), the different sets of reporter signals are associated with different samples and methods wherein the different sets of reporter signals each

comprise the same reporter signals and methods wherein the sets of reporter signals each contain a single reporter signal.

Also disclosed are methods wherein not all of the reporter signals are distinguished and/or separated from molecules lacking the common property, not all of the reporter signals are altered, and not all of the altered forms of the reporter signals are detected at the same time.

Also disclosed are methods wherein all of the reporter signals are distinguished and/or separated from molecules lacking the common property, all of the reporter signals are altered, and all of the altered forms of the reporter signals are detected at different times.

Also disclosed are methods wherein steps (a) through (c) are performed separately for each sample.

B. Reporter Signal Protein Labeling

Also disclosed are methods comprising (a) separating a set of labeled proteins, wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein each reporter signal has a common property, wherein the common property allows the labeled proteins comprising the same protein or peptide to be distinguished and/or separated from molecules lacking the common property, (b) altering the reporter signals, thereby altering the labeled proteins, (c) detecting and distinguishing the altered forms of the labeled proteins from each other.

Also disclosed are methods wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their mass, wherein the altered forms of the labeled proteins are distinguished via differences in the mass-to-charge ratio of the altered forms of the labeled proteins.

Also disclosed are methods wherein the mass of the reporter signals is altered by fragmentation.

Also disclosed are methods wherein alteration of the reporter signals also alters their charge.

Also disclosed are methods wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their charge, wherein the altered forms of the labeled proteins can be distinguished via differences in the mass-to-charge ratio of the altered forms of reporter signals.

Also disclosed are methods wherein the set of labeled proteins comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signals.

Also disclosed are methods wherein the set of labeled proteins comprises ten or more different reporter signals.

Also disclosed are methods wherein the reporter signals are peptides, oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

Also disclosed are methods wherein the reporter signals are coupled to the proteins or peptides.

Also disclosed are methods wherein steps (a) through (c) are performed separately for each labeled protein.

Also disclosed are methods further comprising, prior to step (a), attaching the reporter signals to one or more proteins, one or more peptides, or one or more proteins and peptides.

Also disclosed are methods wherein steps are repeated one or more times using a different set of reporter signals each time.

Also disclosed are methods wherein, prior to step (a), the different sets of reporter signals are attached to proteins or peptides in different samples.

Also disclosed are methods wherein the different sets of reporter signals each comprise the same reporter signals.

Also disclosed are methods wherein the sets of reporter signals each contain a single reporter signal.

Also disclosed are methods wherein not all of the labeled proteins in the set are distinguished and/or separated from molecules lacking the common property, not all of the reporter signals are altered, and not all of the altered forms of the labeled proteins are detected at the same time.

Also disclosed are methods wherein all of the labeled proteins in the set are distinguished and/or separated from molecules lacking the common property, all of the reporter signals are altered, and all of the altered forms of the labeled proteins are detected at different times.

Also disclosed are methods wherein steps (a) through (c) are performed separately for each reporter signal.

Also disclosed are methods wherein the common property is one or more affinity tags associated with the reporter signals.

5 Also disclosed are methods wherein one or more affinity tags are associated with the reporter signals.

Also disclosed are methods wherein the collection of altered forms of the labeled proteins detected constitutes a catalog of proteins.

10 Also disclosed are methods comprising (a) separating one or more labeled proteins, wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein each reporter signal has a common property, wherein the common property allows the labeled proteins comprising the same protein or peptide to be distinguished and/or separated from molecules lacking the common property in each of one or more samples, (b) altering the reporter signals,
15 thereby altering the labeled proteins, (c) detecting and distinguishing the altered forms the labeled proteins from each other.

Also disclosed are methods wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their mass, wherein the altered forms of the labeled proteins are distinguished via differences in the mass-to-charge
20 ratio of the altered forms of labeled proteins.

Also disclosed are methods wherein the mass of the reporter signals is altered by fragmentation.

Also disclosed are methods wherein alteration of the reporter signals also alters their charge.

25 Also disclosed are methods wherein the common property is mass-to-charge ratio, wherein the reporter signals are altered by altering their charge, wherein the altered forms of the labeled proteins can be distinguished via differences in the mass-to-charge ratio of the altered forms of reporter signals.

Also disclosed are methods wherein the reporter signals are coupled to the
30 proteins or peptides.

Also disclosed are methods further comprising, prior to step (a),

attaching the reporter signals to one or more proteins, one or more peptides, or one or more proteins and peptides.

Also disclosed are methods wherein steps are repeated one or more times using a different set of one or more reporter signals each time.

5 Also disclosed are methods wherein, prior to step (a), the different sets of reporter signals are attached to proteins or peptides in different samples.

Also disclosed are methods wherein the different sets of reporter signals each comprise the same reporter signals.

10 Also disclosed are methods wherein the sets of reporter signals each contain a single reporter signal.

Also disclosed are methods wherein not all of the labeled proteins are distinguished and/or separated from molecules lacking the common property, not all of the reporter signals are altered, and not all of the altered forms of the labeled proteins are not detected at the same time.

15 Also disclosed are methods wherein all of the labeled proteins are distinguished and/or separated from molecules lacking the common property, all of the reporter signals are altered, and all of the altered forms of the labeled proteins are detected at different times.

20 Also disclosed are methods wherein steps (a) through (c) are performed separately for each sample and methods wherein the different samples are from the same protein sample and methods wherein the different samples are obtained at different times and methods wherein the different samples are from the same type of organism and methods wherein the different samples are from the same type of tissue and methods wherein the different samples are from the same organism and methods
25 wherein the different samples are obtained at different times.

Also disclosed are methods wherein the different samples are from different organisms. Also disclosed are methods wherein the different samples are from different types of tissues.

30 Also disclosed are methods wherein the different samples are from different species of organisms.

Also disclosed are methods wherein the different samples are from different strains of organisms.

Also disclosed are methods wherein the different samples are from different cellular compartments.

Also disclosed are methods further comprising identifying or preparing proteins or peptides corresponding the proteins or peptides present in one sample but not present
5 in another sample.

Also disclosed are methods further comprising determining the relative amount of proteins or peptides in the different samples.

Also disclosed are methods wherein the common property is one or more affinity tags associated with the reporter signals.

10 Also disclosed are methods wherein one or more affinity tags are associated with the reporter signals.

Also disclosed are methods wherein the pattern of the presence, amount, presence and amount, or absence of labeled proteins in one of the samples constitutes a catalog of proteins in the sample.

15 Also disclosed are methods wherein the pattern of the presence, amount, presence and amount, or absence of labeled proteins in a second one of the samples constitutes a catalog of proteins in the second sample, wherein the catalog of proteins in the first sample is a first catalog and the catalog of proteins in the second sample is a second catalog, the method further comprising comparing the first catalog and the
20 second catalog.

Also disclosed are methods wherein each labeled protein comprises a protein or a peptide and a reporter signal attached to the protein or peptide, wherein the reporter signals comprise peptides, wherein the reporter signal peptides have the same mass-to-charge ratio.

25 Also disclosed are methods wherein the reporter signal peptides have the same amino acid composition.

Also disclosed are methods wherein the reporter signal peptides have the same amino acid sequence.

Also disclosed are methods wherein each reporter signal peptide contains a
30 different distribution of heavy isotopes.

Also disclosed are methods wherein each reporter signal peptide contains a different distribution of substituent groups.

Also disclosed are methods wherein each reporter signal peptide has a different amino acid sequence.

Also disclosed are methods wherein each reporter signal peptide has a labile or scissile bond in a different location.

5 Also disclosed are methods wherein one or more affinity tags are associated with the reporter signals.

Disclosed are methods comprising (a) separating a set of labeled proteins, wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein each labeled protein has a common property,
10 wherein the common property allows the labeled proteins comprising the same protein or peptide to be distinguished and/or separated from molecules lacking the common property, (b) altering the reporter signals, thereby altering the labeled proteins, (c) detecting and distinguishing the altered forms of the labeled proteins from each other.

Disclosed are methods comprising (a) altering labeled proteins, wherein each
15 labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein the labeled proteins are altered by altering the reporter signals, (b) detecting and distinguishing the altered forms of the labeled proteins from each other.

Disclosed are methods of detecting a protein or peptide, the method comprising
20 (a) altering a labeled protein, wherein the labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein the labeled protein is altered by altering the reporter signal, (b) detecting and distinguishing the altered form of the labeled protein from the unaltered form of labeled protein.

Also disclosed are methods further comprising, detecting the unaltered form of
25 labeled protein.

Disclosed are methods of detecting a protein, the methods comprising, detecting a labeled protein, wherein the labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, wherein the labeled protein is altered by altering the reporter signal, detecting an altered form of the labeled protein, wherein
30 the labeled protein is altered by altering the reporter signal, and identifying the protein based on the characteristics of the labeled protein and altered form of the labeled protein.

Also disclosed are methods wherein the labeled protein and altered form of the labeled protein are detected by detecting the mass-to-charge ratio of the labeled protein and the mass-to-charge ratio of the altered form of the labeled protein or the mass-to-charge ratio of the altered form of the reporter signal.

5 Disclosed are methods comprising (a) separating one or more labeled proteins from other molecules, wherein the labeled proteins are derived from one or more samples, wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, (b) altering the reporter signals, thereby altering the labeled proteins, (c) detecting and distinguishing the altered forms the
10 labeled proteins from each other.

Also disclosed are methods further comprising, prior to step (a), associating one or more reporter signals with one or more proteins, one or more peptides, or one or more proteins and peptides from each of the one or more samples.

Also disclosed are methods wherein steps are repeated one or more times using
15 a different set of one or more reporter signals each time.

Also disclosed are methods wherein, prior to step (a), the different sets of reporter signals are attached to proteins or peptides in different samples.

Also disclosed are methods wherein the different sets of reporter signals each comprise the same reporter signals.

20 Also disclosed are methods wherein each reporter signal or each labeled protein has a common property, wherein the common property allows the labeled proteins comprising the same protein or peptide to be distinguished and/or separated from molecules lacking the common property.

Also disclosed are methods wherein the one or more labeled proteins are
25 derived from a single sample.

Also disclosed are methods wherein a single labeled protein is distinguished and/or separated from other molecules.

Also disclosed are methods wherein a plurality of labeled proteins are distinguished and/or separated from other molecules.

30 Also disclosed are methods wherein the detected altered forms of the labeled proteins constitute a catalog of proteins in the sample.

Also disclosed are methods wherein one or more labeled proteins are derived from each of a plurality of samples.

Also disclosed are methods wherein a single labeled protein derived from each of the samples is distinguished and/or separated from other molecules.

5 Also disclosed are methods wherein a plurality of labeled proteins derived from each of the samples are distinguished and/or separated from other molecules.

Also disclosed are methods wherein the detected altered forms of the labeled proteins derived from each sample constitute a catalog of proteins in the sample.

10 Disclosed are catalogs of proteins and peptides comprising, proteins and peptides in a sample detected by (a) separating one or more labeled proteins from other molecules, wherein the labeled proteins are derived from the sample, wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, (b) altering the reporter signals, thereby altering the labeled proteins, (c) detecting and distinguishing the altered forms the labeled proteins from each other.

15 Also disclosed are catalogs of proteins and peptides comprising, proteins and peptides in one or more samples detected by (a) separating one or more labeled proteins from other molecules, wherein the labeled proteins are derived from the one or more samples, wherein each labeled protein comprises a protein or peptide and a reporter signal attached to the protein or peptide, (b) altering the reporter signals, thereby
20 altering the labeled proteins, (c) detecting and distinguishing the altered forms the labeled proteins from each other.

C. Reporter Signal Calibration

Disclosed are methods method of producing a protein signature, the method comprising (a) treating a protein sample to produce protein fragments, wherein the
25 protein fragments comprise a set of target protein fragments, wherein the target protein fragments can be altered, wherein the altered forms of the target protein fragments can be distinguished from the other altered forms of the target protein fragments, (b) mixing the target protein fragments with a set of reporter signal calibrators, wherein each target protein fragment shares a common property with at least one of the reporter signal
30 calibrators, wherein the common property allows the target protein fragments and reporter signal calibrators having the common property to be distinguished and/or separated from molecules lacking the common property, wherein the target protein

fragment and reporter signal calibrator that share a common property correspond to each other, wherein the reporter signal calibrators can be altered, wherein the altered form of each reporter signal calibrator can be distinguished from the altered form of the target protein fragment with which the reporter signal calibrator shares a common
5 property, (c) separating the target protein fragments and reporter signal calibrators from other molecules based on the common properties of the target protein fragments and reporter signal calibrators, (d) altering the target protein fragments and reporter signal calibrators, (e) detecting the altered forms of the target protein fragments and reporter signal calibrators, wherein the presence, absence, amount, or presence and amount of
10 the altered forms of the target protein fragments indicates the presence, absence, amount, or presence and amount in the protein sample of the target protein fragments from which the altered forms of the target protein fragments are derived, wherein the presence, absence, amount, or presence and amount of the target protein fragments in the protein sample constitutes a protein signature of the protein sample.

15 Also disclosed are methods wherein steps (d) and (e) are performed simultaneously.

Also disclosed are methods wherein the altered forms of the target protein fragments are detecting using mass spectrometry.

20 Also disclosed are methods wherein steps (c), (d), and (e) are performed with a tandem mass spectrometer.

Also disclosed are methods wherein the tandem mass spectrometer comprises a first stage and a last stage, wherein step (c) is performed using the first stage of the tandem mass spectrometer to select ions in a narrow mass-to-charge range, wherein step (d) is performed by collision with a gas, and wherein step (e) is performed using
25 the final stage of the tandem mass spectrometer.

Also disclosed are methods where the first stage of the tandem mass spectrometer is a quadrupole mass filter and methods where the final stage of the tandem mass spectrometer is a time of flight analyzer and methods wherein the final stage of the tandem mass spectrometer is a time of flight analyzer.

30 Also disclosed are methods wherein the mass-to-charge range is varied to cover the mass-to-charge ratio of each of the target protein fragments.

Also disclosed are methods wherein a predetermined amount of each reporter signal calibrator is mixed with the target protein fragments, wherein the amount of each altered form of reporter signal calibrator detected provides a standard for assessing the amount of the altered form of the corresponding target protein fragment.

5 Also disclosed are methods wherein the amount of at least two of the reporter signal calibrators is different.

Also disclosed are methods wherein the relative amount each reporter signal calibrator is based on the relative amount of each corresponding target protein fragment expected to be in the protein sample.

10 Also disclosed are methods wherein the amount of each of the reporter signal calibrators is the same.

Also disclosed are methods wherein the target protein fragments and reporter signal calibrators are altered by fragmentation.

15 Also disclosed are methods wherein the target protein fragments and reporter signal calibrators are altered by cleavage at a photocleavable amino acid.

Also disclosed are methods wherein the target protein fragments and reporter signal calibrators are fragmented in a collision cell.

Also disclosed are methods wherein the target protein fragments are fragmented at an asparagine-proline bond.

20 Also disclosed are methods wherein the protein fragments are produced by protease digestion of the protein sample.

Also disclosed are methods wherein the protein fragments are produced by digestion of the protein sample with a serine protease.

Also disclosed are methods wherein the serine protease is trypsin.

25 Also disclosed are methods wherein the protein fragments are produced by digestion of the protein sample with Factor Xa or Enterokinase.

Also disclosed are methods wherein the protein fragments are produced by cleavage at a photocleavable amino acid.

30 Also disclosed are methods wherein the common property is mass-to-charge ratio, wherein the target protein fragments and reporter signal calibrators are altered by altering their mass, their charge, or their mass and charge, wherein the altered forms of the target protein fragments and reporter signal calibrators can be distinguished via

differences in the mass-to-charge ratio of the altered forms of the target protein fragments and reporter signal calibrators.

Also disclosed are methods wherein the set of target protein fragments comprises two or more, three or more, four or more, five or more, six or more, seven or
5 more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different target protein fragments.

Also disclosed are methods wherein the set of target protein fragments comprises ten or more different target protein fragments.

10 Also disclosed are methods wherein the set of reporter signal calibrators comprises two or more, three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, thirty or more, forty or more, fifty or more, sixty or more, seventy or more, eighty or more, ninety or more, or one hundred or more different reporter signal calibrators.

15 Also disclosed are methods wherein the reporter signal calibrators comprise peptides, wherein the peptides have the same mass-to-charge ratio as the corresponding target protein fragments.

Also disclosed are methods wherein the peptides have the same amino acid composition as the corresponding target protein fragments and methods wherein the
20 peptides have the same amino acid sequence as the corresponding target protein fragments and methods wherein each peptide has a different amino acid sequence than the corresponding target protein fragment and methods wherein each peptide has a labile or scissile bond in a different location.

Also disclosed are methods wherein the reporter signal calibrators are peptides,
25 oligonucleotides, carbohydrates, polymers, oligopeptides, or peptide nucleic acids.

Also disclosed are methods further comprising comparing the protein signature to one or more other protein signatures.

Also disclosed are methods wherein at least one of the target protein fragments comprises at least one modified amino acid.

30 Also disclosed are methods wherein the modified amino acid is a phosphorylated amino acid, an acylated amino acid, or a glycosylated amino acid.

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